Characterisation of an Ubiquitin Binding CUE Domain in Presenilin-1

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by

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Declaration

This thesis has not been previously submitted, in part or in whole, to this or any other

University for any degree and is, unless otherwise stated, the original work of the author.

Signed: _____

Stephen Duggan

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Abstract

The presenilins are the catalytic component of the gamma-secretase protease complex, involved in the regulated intramembrane proteolysis of numerous type-1 transmembrane proteins, including Amyloid precursor protein (APP) and Notch. In addition to their role in the γ-secretase complex the presenilins are involved in a number of γ-secretase independent functions such as calcium homeostasis, apoptosis, inflammation and protein trafficking. Presenilin function is known to be regulated through posttranslational modifications like endoproteolysis, phosphorylation and ubiquitination. Using a bioinformatics and protein sequence analysis approach this lab has identified a putative ubiquitin binding CUE domain in the presenilins.

The aim of this project was to characterise the function of the presenilin CUE domains. Firstly, the presenilins are shown to contain a functional ubiquitin-binding CUE domain that preferentially binds to K63-linked polyubiquitin chains. The PS1 CUE domain is shown to be dispensable for PS1 endoproteolysis and γ-secretase mediated cleavage of APP, Notch and IL-1R1. This suggests the PS1 CUE domain is involved in a γ-secretase independent PS1 function.

Our hypothesis is that the PS1 CUE domain is involved in regulating PS1's intermolecular protein-protein interactions or intramolecular PS1:PS1 interactions. Here the PS1 CUE domain is shown to be dispensable for the interaction of PS1 and the K63-linked polyubiquitinated PS1 interacting proteins $P75^{NTR}$, IL-1R1, TRAF6, TRAF2 and RIP1. To further investigate PS1 CUE domain function a mass spectrometry proteomics based approach is used to identify PS1 CUE domain interacting proteins. This proteomics approach demonstrated that the PS1 CUE domain is not required for PS1 dimerization. Instead a number of proteins that

interact with the PS1 CUE domain are identified as well as proteins whose interaction with PS1 is downregulated by the presence of the PS1 CUE domain. Bioinformatic analysis of these proteins suggests possible roles for the PS1 CUE domain in regulating cell signalling, ubiquitination or cellular trafficking.

Abbreviations

Aβ	Amyloid β
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
AICD	APP intracellular domain
Aph-1	Anterior pharynx-defective 1
APP	Amyloid precursor protein
AV	Autophagic vacuoles
BACE	β-site APP-cleaving enzyme 1
BCA	Bicinchoninic acid
βTrCP2	β-transducin repeat protein 2
CDK5	Cyclin-dependent kinase 5
CHO	Chinese hamster ovary
CHX	Cycloheximide
CpdE	Compound E
CREB	cAMP response element binding
CTF	C-terminal fragment
CUE	Coupling of ubiquitin to ER degradation
DAPT	N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester

DISC	Death-inducing signalling complex
DR	Death receptor
DUB	Deubiquitinase
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease
FAT	Fast axonal transport
FBXL12	F-box/LRR-repeat protein 12
GCDH	Glutaryl-CoA dehydrogenase
GFAP	Glial fibrillary acidic protein
GSI	γ-secretase inhibitor
GSK3β	Glycogen synthase kinase 3 β
GSM	γ-secretase modulator
HEK293T	Human embryonic kidney 293T
ICD	Intracellular domain
IL	Interleukin
InsP3R	Inositol triphosphate receptor

IP	Immunoprecipitation
JNK	c-Jun N-terminal kinases
LB	Luria broth
LTP	Long term potentiation
LUBAC	Linear ubiquitin chain assembly complex
MALDI-TOF	Matrix assisted laser desorption/ionisation time-of-flight
MEF	Murine embryonic fibroblasts
MS	Mass spectrometry
Nct	Nicastrin
NICD	Notch intracellular domain
NSAID	Non-steroidal anti-inflammatory drugs
NTF	N-terminal fragment
PDCD4	Programmed cell death 4
PDGF	Platelet-derived growth factor
Pen-2	Presenilin enhancer 2
PIP2	Phosphatidylinositol4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbal 12-myristate 13-acetate

PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
PS1	Presenilin-1
PS2	Presenilin-2
PSAP	Presenilin-1 associated protein
PScDKO	Presenilin conditional double knockout
PSDKO	Presenilin double knock out
PSH	Presenilin homologue
RPN1	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1
RyR	Ryanodine receptor
SCAM	Substituted-cysteine accessibility method
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERCA	Sarcoendoplasmic reticulum calcium transport ATPase
SLFN11	Schlafen family member 11
SMIM4	Small integral membrane protein 4
TACE	TNF-α converting enzyme
TAP	Tandem affinity purification
TβR1	TGFβ Type 1 Receptor

TBS	Tris buffered saline
TIM50	Mitochondrial import inner membrane translocase subunit
TMD	Transmembrane domain
TNF-α	Tumour necrosis factor α
TNFR1	TNF receptor 1
TRADD	TNFR associated death domain
TRAF5	TNF receptor associated factor 5
TRAF6	TNF receptor associated factor 6
TV	Transcript variant
Ub	Ubiquitin
UBA	Ubiquitin associated domain
Ubc	Ubiquitin conjugating
UBD	Ubiquitin binding domain
UBL	Ubiquitin like moiety
UIM	Ubiquitin interacting motif
UPS	Ubiquitin proteasome system

.

Chapter 1 Introduction

Part of this section has been previously published by Cellular Signalling [Duggan S.P. and McCarthy J.V. (2016)] “Beyond γ-secretase activity: The multifunctional nature of presenilins in cell signalling pathways.” Cell. Signal. 016 Jan;28(1):1-11. doi: 10.1016/j.cellsig.2015.10.006.

1.1 Regulated Intramembrane Proteolysis

Presenilin 1 (PS1) and presenilin 2 (PS2) were discovered as part of genetic studies related to familial Alzheimer's disease (FAD) and to date over 200 mutations in PS1 and 10 mutations in PS2 have been associated with the disease [Zou *et al* 2014]. Alzheimer's disease (AD) is characterised pathogenically by the formation of neurofibullar tangles arising from hyperphosphorylated Tau protein and by amyloid plaque deposits formed from aggregations of amyloid β (Aβ) peptides [Zou *et al* 2014]. Aβ peptides are formed from the proteolytic cleavage of the transmembrane spanning amyloid precursor protein (APP) holoprotein. APP is sequentially cleaved: first by either α-secretase or β-secretase (BACE) and then by γ-secretase. PS1 and PS2 form part of the γ-secretase multi-protein complexes, consisting of the presenilins, anterior pharynx-defective 1 (Aph-1), presenilin enhancer 2 (Pen-2) and Nicastrin (Nct) [Spasic and Annaert 2008]. To date more than 100 γ-secretase substrates have been identified including APP and a large number of cell surface receptors including Notch [Haapasalo *et al* 2011; McCarthy *et al* 2009].

The sequential proteolysis of APP culminating in intramembrane cleavage by γ-secretase now serves as a model for the regulated intramembrane proteolysis of numerous transmembrane proteins **(Figure 1.1)** [Brown *et al* 2000; Medina and Dotti 2003]. Cleavage of APP by α-secretase or β-secretase results in the release of respective soluble ectodomains (sAPPα or sAPPβ), generation of the APP intracellular domain (AICD) and P3 or Aβ peptides that represent the remaining transmembrane domain of APP [Mills and Reiner 1999]. The sAPPα and sAPPβ fragments have been shown to promote neurite growth in cortical neurons in rats and mice and to regulate cell-signalling pathways [Hasebe *et al* 2013; Ohsawa *et al* 1997; Hartl *et al* 2013]. The AICD fragment has been shown to interact with c-ABL

and Fe65 to regulate gene transcription [Vazquez *et al* 2009], in particular in regulating the transcription of neprilysin [Grimm *et al* 2015]. In addition the AICD is involved in cellular functions such as regulating Wnt signalling and apoptosis [Zhou *et al* 2012; Wang *et al* 2014].

In general, substrates that undergo regulated intramembrane proteolysis are initially cleaved in the extracellular domain by sheddases such as TACE (TNFα converting enzyme) or ADAM (a disintegrin and metalloproteinase domain)/α-secretase, or by aspartyl proteases, such as BACE/β-secretase, before cleavage by the I-CLiP family of proteases [Mills and Reiner 1999; Langosch *et al* 2015]. Furthermore, it has been reported that prerequisite ectodomain shedding may not be required for subsequent cleavage by γ-secretase for some substrates [Laurent *et al* 2015].

For several substrates γ-secretase cleavage plays an essential role in a signalling paradigm whereby generation of intracellular domains (ICDs) allows for the spatial segregation of divergent signalling pathways or as is the case for Notch, allows for the translocation of ICDs to the nucleus where they enable transcriptional activation activity [Jurisch-Yaksi *et al* 2013; Medina and Dotti 2003]. Indeed, *Psen1* knockout animals display a predominant Notch loss of function phenotype resulting from loss of γ-secretase cleavage of Notch, highlighting the importance of γ-secretase in Notch signalling [De Strooper *et al* 1999; Geling *et al* 2002; Micchelli *et al* 2003]. Notch cleavage by γ-secretase has been shown to regulate diverse cellular functions including epithelial differentiation in the airway [Gomi *et al* 2015] and in memory formation in rats [Conboy *et al* 2007]. Similarly the γ-secretase mediated cleavage of ErbB4 has been to result in the creation of an ICD that translocates to the nucleus [Vidal *et al* 2005] and which regulates alveolar cell differentiation and lung maturation [Fiaturi *et al* 2014; Hoeing *et al* 2011]. Meanwhile, the ICD of the

$P75^{NTR}$ has been shown to negatively regulate cell invasiveness in glioblastoma cell lines [Berghoff *et al* 2015]. Cleavage can also be used as a signal for degradation of transmembrane protein fragments and the maintenance of so-called 'membrane proteostasis' [Lichtenthaler *et al* 2011].

PS1 and PS2 are integral transmembrane proteins which both contain 9 transmembrane (TMDs) and a large intracellular loop between TMDs 6 and 7 [Oh and Turner 2005] **(Figure 1.2)**. PS1 has been shown to be both located and active within the endoplasmic reticulum (ER) and early stage of the Golgi of the cell [Annaert *et al* 1999] suggesting that this represents the location of the γ-secretase complexes. PS1 and PS2 undergo endoproteolysis in the loop domain before reaching maturity forming the N-terminal fragment (NTF) and C-terminal fragment (CTF) heterodimers and this process occurs in acidic cellular compartments after passing through the ER [Kim *et al* 2007]. The presenilin proteins are aspartyl proteases and act as the enzyme component of γ-secretase. Their active site is present in the sixth and seventh transmembrane at residues Asp257 and Asp385 in PS1 [Wolfe *et al* 1999] and Asp263 and Asp366 in PS2 [Kimberly *et al* 2000]. Cysteine residues within TMD 1 and TMD 8 [Kornilova *et al* 2006] and a C-terminal PAL domain in TMD 9 of presenilin [Wang *et al* 2006] also contribute towards the structure of the presenilin active site [Spasic and Annaert 2008].

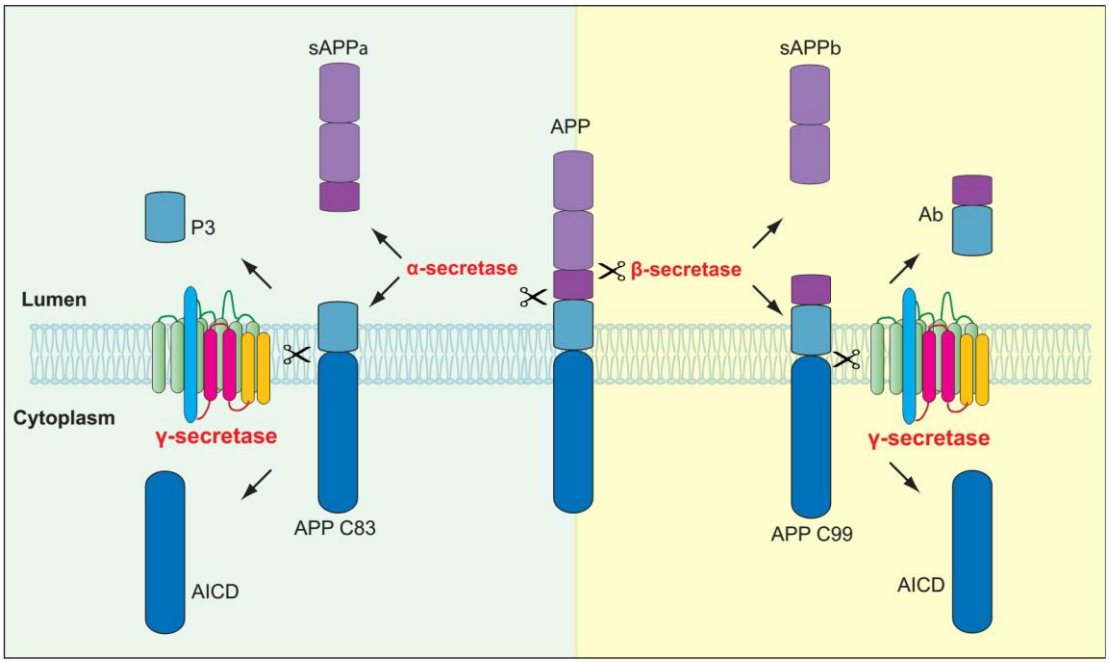


Figure 1.1 Schematic of the Regulated Intramembrane Proteolysis of APP. This schematic shows the alternative cleavage of APP by either the α-secretase (left) or β-secretase (right) pathways. Firstly in the respective pathways the APP ectodomain is cleaved to release either sAPPα or sAPPβ into the lumen, leaving the APP C83 or APP C99 membrane bound C-terminal fragments. These membrane bound fragments are then cleaved by the γ-secretase complex to release the AICD into the cytoplasm and the P3 or Aβ fragments into the lumen by either the α-secretase or β-secretase pathways, respectively. This figure was taken from [Duggan and McCarthy 2016]**.**

1.2 Presenilins, Post-Translational Modification and Cell Signalling

The presenilins undergo a number of post-translational modification including endoproteolysis, caspase cleavage [Walter *et al* 1999], phosphorylation [Kirschenbaum *et al* 2001; Fluhrer *et al* 2004; Kuo *et al* 2008; Lau *et al* 2002] and ubiquitination [Yan *et al* 2013; Gudey *et al* 2014; Li *et al* 2002] **(Figure 1.2)**. PS1 can be phosphorylated at a number of different serine or threonine residues by proteins such as protein kinase A (PKA) [Fluhrer *et al* 2004], protein kinase C (PKC) [Walter *et al* 1999], glycogen synthase kinase 3β (GSK3β) [Kirschenbaum *et al* 2001], c-Jun N-terminal kinases (JNK) [Kuo *et al* 2008] and cyclin dependent kinase 5 (CDK5) [Lau *et al* 2002]. PS1 can be phosphorylated in the intracellular loop by both PKA (at Ser310) and by PKC (at Ser346) [Fluhrer *et al* 2004]. The phosphorylation of the presenilins by these kinases can result in alterations in γ-secretase activity [Kuo *et al* 2008], β-catenin signalling [Uemura *et al* 2007] and presenilin stability [Lau *et al* 2002]. The PKC phosphorylation site is located near the caspase cleavage site in PS1 and pseudo-phosphorylation at this site inhibits caspase-3 related cleavage of PS1 in vitro and during induced apoptosis in HEK293T cells. Similarly, pseudo-phosphorylation near the caspase cleavage site, at residues Ser327 and Ser330, prevents caspase cleavage in PS2 [Walter *et al* 1999]. Tumour necrosis factor α (TNF-α) upregulates γ-secretase activity in a JNK dependent manner; JNK phosphorylates PS1 at residues Ser319 and Thr320 and mutations at these residues removes the ability of TNF-α to upregulate γ-secretase activity [Kuo *et al* 2008]. JNK phosphorylation at these residues also increases the stability of the PS1 CTF relative to that of the NTF. Similarly, CDK5 phosphorylation at Thr354 has been to increase the stability of PS1 CTF [Lau *et al* 2002].

Figure 1.2 Posttranslational Modifications of the Presenilin CUE and Intracellular Loop Domains. Schematic showing the CUE domains of the presenilins are highlighted in red and the remainder of the presenilin intracellular loop domains are highlighted in green. The area deleted in the PS1ΔE9 mutation is marked by ΔE9. Previously described posttranslational modifications of the intracellular loop domains of human presenilins, PS1 and PS2 are also shown. PS1 contains three GSK3β phosphorylation sites [underlined] whereas PS2 contains none. PS1 and PS2 are subject to serine/threonine phosphorylation by PKA [●], PKC [○], CDK5 [■], GSK3β [□], JNK [◊] AND CK1/2 [*] and mapped to specific individual residues. PS1 is also ubiquitinated by Fbw7/Sel-10 and TRAF6 but the site(s) have not been mapped to individual residues. The TRAF6-binding domain (boxed in grey) and the endoproteolysis and caspase cleavage sites are also indicated [arrows]. Schematic showing known familial Alzheimer's disease mutations within the CUE domains of the presenilins. Mutations are underlined/highlighted in black This figure was taken from [Duggan and McCarthy 2016].

PS1 is ubiquitinated by the E3 ligases SEL-10 [Li *et al* 2002] and tumour necrosis factor receptor associated factor 6 (TRAF6) [Yan *et al* 2013; Gudey *et al* 2014]. The ubiquitination of PS1 by SEL-10 reduces the level of PS1 by targeting PS1 for degradation through the ubiquitin-proteasome system [Li *et al* 2002]. However while the overexpression of SEL-10 in HEK293T cells caused a reduction in levels of PS1 it also leads to an increase in the levels of Aβ detected. SEL-10 has also been shown to down regulate the signalling activity of the PS1 substrate Notch [Li *et al* 2002]. PS1 and Fbw7, the mammalian homolog of Sel10, have been shown to interact to regulate the stability and activity of the epidermal growth factor receptor (EGFR) and the Notch ICD (NICD) [Rocher-Ros *et al* 2010]. The authors suggest that PS1 and Fbw7 interact with one another in a negative feedback loop to control the levels of EFGR and Notch respectively, which may play a role in skin carcinogenesis. Ubiquilin is a protein which contains both an ubiquitin like domain (UBL) and an ubiquitin associated (UBA) domain. The ubiquilin UBA domain has been shown bind to both PS1 and PS2 in GST pull down assays [Mah *et al* 2000]. Overexpression of ubiquilin has been shown to reduce the formation of the NTF and CTF of the presenilin proteins by stabilising the level of the full length presenilin proteins in the cell [Massey *et al* 2005]. Ubiquilin transcript variants (TV) 1 and 3 have been shown to cause the accumulation of higher molecular weight forms of PS1 (HMW-PS1) [Viswanathan *et al* 2011]. Ubiquilin is hypothesised to act as a shuttle between ubiquitinated proteins and the proteasome and it has been suggested that the accumulation of HMW-PS1 is a result of weak interactions between Ubiquilin-1 TV3 and the proteasome [Viswanathan *et al* 2011].

The presenilin proteins contain TRAF6-binding sequence which allows for the interaction between the E3 ligase TRAF6 and the presenilin proteins [Elzinga *et al*

2009; Powell *et al* 2009] and by doing so TRAF6 induces polyubiquitination of PS1 [Yan *et al* 2013; Gudey *et al* 2014]. TRAF6 has been shown to extend the half-life of full length PS1 without having an effect on γ-secretase activity [Yan *et al* 2013]. The interaction between TRAF6 and PS1 promotes the cleavage of the γ-secretase substrates TGFβ Type 1 Receptor (TβR1) [Gudey *et al* 2014] and $P75^{NTR}$ [Powell *et al* 2009] and the loss of the E3 ligase activity of TRAF6 abolishes the TRAF6 mediated cleavage of $P75^{NTR}$. These data suggest that the interaction between TRAF6, PS1 and γ-secretase substrates plays a role in modulating γ-secretase activity. However, as can be in the case of $P75^{NTR}$, TRAF6 can ubiquitinate both PS1 and some γ-secretase substrates, therefore the exact role played by TRAF6 mediated ubiquitination of PS1 remains to be fully elucidated [Gudey *et al* 2014]. All of this suggests that the interactions between presenilin, ubiquitin, E3 ligases such as TRAF6 and ubiquitin binding proteins such as ubiquilin play a role in regulating both the γ-secretase-dependent and –independent functions of presenilin.

1.3 Ubiquitin, the Ubiquitin Proteasome System and Ubiquitin Binding Domains

Ubiquitin is a 76 amino acid protein that is covalently bonded to target proteins at lysine residues as a post-translational modification [Komander and Rape 2012]. The transfer of ubiquitin to target proteins is controlled by a series of proteins known as the ubiquitin activating E1, the ubiquitin conjugating (Ubc) E2 and the ubiquitin ligase E3. Ubiquitin is first bound to the E1 protein before it is transferred to the E2 protein; the E3 ligase then connects the E2 and the target protein thus binding ubiquitin to its target **(Figure 1.3)** [Welchman et al 2005]. There are estimated to be two E1, roughly forty E2 and over six hundred E3 proteins present in eukaryotes [Grabbe *et al* 2011]; the variety within these proteins is what allows ubiquitin to be

targeted to such a large range of proteins and for the diversity of uibiquitin linkages. There are a number of deubiquitylating enzymes (DUBs) found in eukaryotes, which play a role in down regulating ubiquitin signalling [Grabbe *et al* 2011]. Ubiquitin contains seven different lysine residues (K6, K11, K27, K29, K33, K48 and K63) [Chen and Sun 2009] all of which can be used to form polyubiquitin chains; these ubiquitin chains can be made up of mixed lysine linkages or form forked ubiquitin chains. The lysine residues of ubiquitin can covalently bond to the C-terminal glycine residue of another ubiquitin molecule to form polyubiquitin chains of varying lengths. Linear ubiquitin chains can also be formed between the N-terminal methionine and the C-terminal glycine residues [Grabbe *et al* 2011].

The ubiquitin proteasome system (UPS) is the cellular system in which ubiquitin proteins are bonded to proteins thus targeting them for degradation in the proteasome. The targeting of proteins to the UPS is determined by the binding of K48-linked polyubiquitin chains to UPS substrates [Pickart 2000]. Further research has provided insight into the formation and structure of polyubiquitin chains through the other lysine residues of ubiquitin [Komander and Rape 2012]. Unlike K48-linked ubiquitination the effects of K63-linked ubiquitination are more varied in outcome. Mutations at the K63 residue of ubiquitin in *S. cerevisiae* have been shown to have no negative affect on the degradative pathways in the yeast [Spence et al 1995]. However, *S. cerevisiae* strains containing this mutation were less resistant to treatment with UV or alkylating agents, showing that K63-linked ubiquitin chains

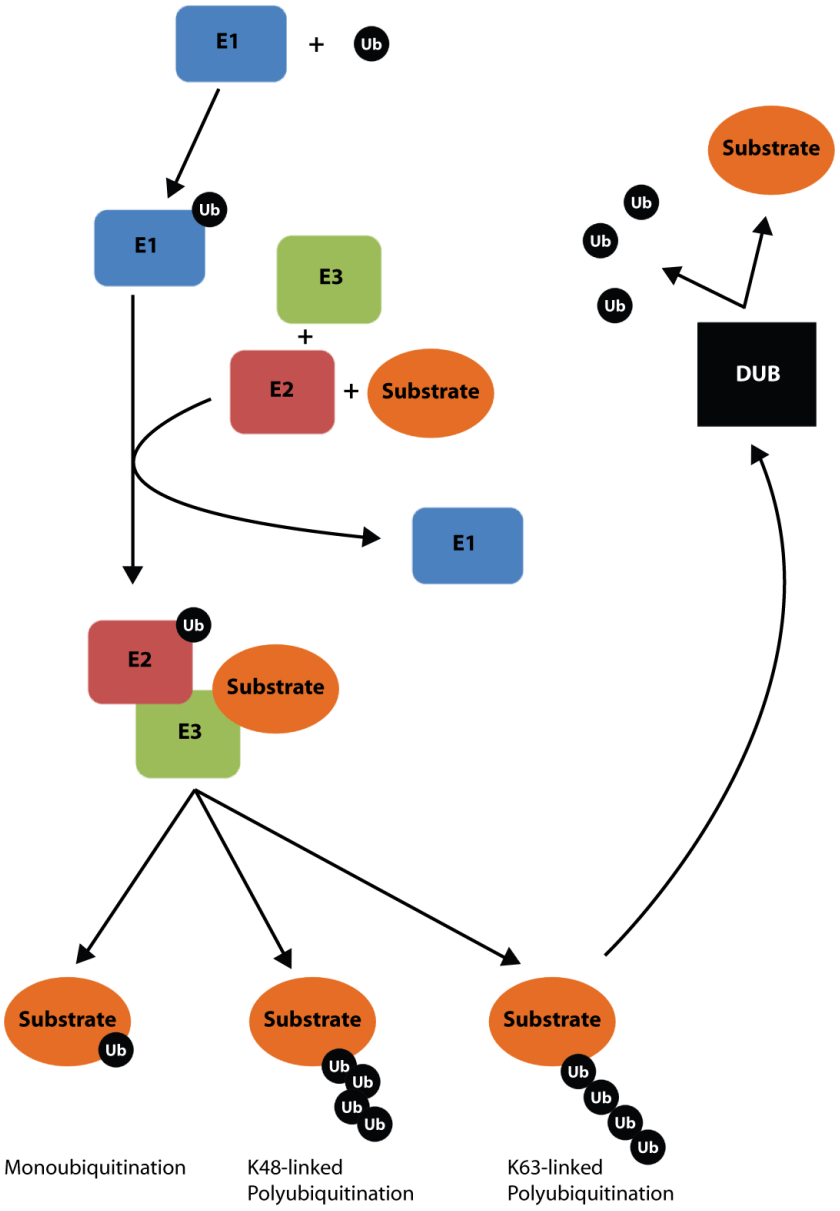


Figure 1.3 Schematic of the E1/E2/E3 ubiquitination system. Firstly the ubiquitin activating E1 enzyme binds to an ubiquitin molecule (Ub). Next the E1-Ub complex interacts with an E2 ubiquitin activating enzyme to transfers the Ub molecule to the E2 protein. Finally the E3 ubiquitin ligase interacts with both the E2-Ub complex and the substrate to transfer the Ub molecule to the substrate. The substrate can undergo both mono- and poly-ubiquitination with the closed confirmation of K48-linked polyubiquitination and the open confirmation of K63-linked polyubiquitination shown here. Deubiquitinases (DUBs) remove Ub molecules from their substrates to release free Ub molecules and deubiquitinated substrates.

play some role in DNA repair. K63-linked ubiquitination can also decrease the level of targeted protein via a non-proteasomal route. Activation of the interleukin-1 (IL-1) receptor, type 1 (IL-1R1) leads to K63-linked polyubiquitination of IRAK1 leading to a decrease in IRAK1 levels. However, this decrease in IRAK1 levels is not affected by proteasome inhibition but occurs via another cellular process [Windheim et al 2008]. K63-linked polyubiquitin of IκB Kinase has been shown to up-regulate the NFκB pathway [Deng et al 2000] and thus have an effect on regulating gene transcription. Additionally, TNF receptor associated factor 5 (TRAF5) mediated K63-linked ubiquitination of retinoid-related orphan nuclear receptor γt has recently been shown to promote gene transcription [Wang *et al* 2015]. The linear ubiquitin chain assembly complex (LUBAC) has been shown to positively regulate NF-κB signalling through NEMO [Tokunaga *et al* 2009]. The LUBAC complex causes the formation of linear ubiquitin chains, where the primary methionine residue is bound to the terminal glycine residue, on to its substrates. A20 has been shown to inhibit the interactions between NEMO and LUBAC to negatively regulate NF-κB signalling [Verhelst *et al* 2012]. This demonstrates how cellular signalling pathways can be affected through the ligation of differing polyubiquitin chains. These differences in the ubiquitination state of proteins can be detected through the presence of distinct ubiquitin binding domains within interacting proteins [Komander and Rape 2012].

1.4 Ubiquitin Binding Domains

Ubiquitin binding domains (UBDs) are the means through which a protein's ubiquitination state can be recognised to regulate downstream cell signalling events [Chen and Sun 2009]. A number of UBDs have been found, including the ubiquitin interacting motif (UIM), the ubiquitin associated (UBA) and the coupling of

ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domains amongst others **(Table 1.1)** [reviewed in Chen and Sun 2009]. The importance of UBDs in regulating cell signalling can be seen in the NFκB pathway, where several proteins are known to contain UBDs required for signal propagation [Chen 2005]. The UBA and CUE domains are similar in size, roughly 40 residues, and show a structural homology. Both the UBA and CUE domains share a three helical secondary structure and both can interact with a hydrophobic patch on the surface of ubiquitin around the Ile44 residue [Hurley et al 2006]. Some UBA domains have shown an ability to bind both the K48- and K63-linked polyubiquitin chains [Matta-Camacho *et al* 2009] while others, such as K63-linked polyubiquitin binding UBA domain of the sequestosome1/P62 protein [Seibenhener *et al* 2004], have shown a preference for binding to particular polyubiquitin chains. The UBA domain of P62 has been shown to regulate the turnover of proteins via the ubiquitin-proteasome system. Mutations within the UBA domain of sequestosome1/P62 are associated with Paget's disease [Seibenhener *et al* 2004] indicating that the ubiquitin binding properties of sequestosome1/P62 play an important physiological role. The CUE domain was discovered as part of a bioinformatics search for sequence homology with the yeast protein Cue1p, which recruits other proteins to the ER membrane [Ponting 2000]. This research showed a number of conserved residues, an invariant proline residue in particular, but also includes a number of semi-conserved sequences such as the phenylalanine-proline and di-leucine sequences that were shared amongst the examined proteins [Ponting 2000]. Studies of the CUE domain containing yeast protein Vps9 showed that the FP and LL motifs of the Vps9 CUE domain form part of the ubiquitin binding surface [Shih *et al* 2003]. In this study mutations at residues at F420 and P421 of the Vps9 CUE domain caused the loss or

reduction of the ubiquitin binding properties of the protein demonstrating the importance of this motif for the ubiquitin binding function of the Vps9 CUE domain. A structural study of the Vps9 CUE domain showed that the FP motif and the second leucine (L447) of the di-leucine motif directly interacted with the surface of ubiquitin molecules, while the first leucine (L446) residue of the Vps9 di-leucine motif played a role in stabilising the structure of the domain [Prag *et al* 2003].

Table 1.1 List of known ubiquitin binding domains.

(Adapted from Dikic *et al* 2009)

Ub-binding domain	Protein	Function	Reference
α-helical type			
CUE	Vps9, Cue1p	Trafficking, ERAD	Shideler et al 2015; Bagola et al 2013
GAT	GGA3	Trafficking	Bilodeau et al 2004
MIU	Rabex-5	Cell signalling	Sokratous et al 2012
UBA	p62, ubiquilin	proteasomal degradation, autphagy	Seibenhener et al 2004; Rothenburg et al 2010
UBAN	NEMO	Cell signalling	Wagner et al 2008
UBM	TLS polymerase ι	DNA replication	Kannouche et al 2005
UIM	Eps15	Trafficking	Gucwa et al 2014
VHS	STAM1	Lysosomal degradation	Lim et al 2011
Zinc Finger			
A20 ZnF	A20	cell signalling	Bellail et al 2012

NZF	HOIL-1L	cell signalling	Sato et al 2011
UBZ	TLS polymerase ι	DNA replication	Kannouche et al 2005
ZnF UBP	isopeptidase -T	deubiquitinase activity	Sokratous et al 2012
Ubiquitin-conjugating like domain			
UEV	Vps23	Trafficking	Blanchin-Roland 2011
Ubc	UbcH5	Ubiquitin conjugation	Yuhuang et al 2014
Plekstrin Homology domain			
PRU	Rpn13	Proteasomal degradation	Aguileta et al 2015
GLUE	EAP45	Trafficking	Alam et al 2006
Other			
Jab1/MPN	Prp8p	Spliceosome	Bellare et al 2006
PFU	Doa1	Trafficking	Mullally et al 2006
SH3	Sla1, CIN85	cell signalling	Stamenova et al 2007

UBDs have been shown to regulate protein function by competitive or non-competitive binding with other interacting partners. The interaction between ubiquitin and different CUE domains can be involved in regulating the ubiquitination of other proteins or in regulating self-ubiquitination **(Table 1.2)**. For example the CUE domain of the E3 ligase GP78 has been shown to be involved in regulating the length of polyubiquitin chains that GP78 builds up on its substrates [Liu *et al* 2014]. The GP78 CUE domain non-competitively binds to the incipient K48-linked polyubiquitin chain as it is synthesized onto the substrate thus stabilizing the chain and allowing further extension. Similarly, functional CUE domains have been shown

to be required for regulating a protein's ubiquitination state. Mono-ubiquitination of Vps9 is eliminated by deletion of the proteins CUE domain [Shih *et al* 2003], suggesting that the Vps9 CUE domain interacts with components of the cell's ubiquitination system. On the other hand in Tollip ubiquitin not only binds to the proteins CUE domain but also interacts with the protein's lipid binding C2 domain [Mitra *et al* 2013], showing that there is competitive binding of ubiquitin or phosphoinositide to Tollip. This competition between the two protein domains regulates the association of Tollip with the membrane. These studies demonstrate that the ubiquitin binding function of the CUE domain is involved in self-ubiquitination and the ubiquitination of other proteins. Additionally, these interactions can influence the cellular localization of the protein. Vps9 shows a preference for binding K48 linked polyubiquitin and both Vps9 and its human homolog Rabex-5 are involved in trafficking to the lysosome [Donaldson *et al* 2003]. The CUE domain of CUEDC2 has been shown to be essential for the deactivation of the progesterone receptor via the ubiquitin-proteasome pathway [Zhang *et al* 2007]. As shown here CUE domains, and other ubiquitin binding domains, can confer a variety of functions upon a protein and be involved in diverse cellular functions.

Table 1.2 List of known CUE domain containing proteins.

Protein	CUE domain function	Reference
Tollip	Autophagy	Lu et al 2014
Vps9	Endocytosis	Davies et al 2003
Fun30	chromatin remodelling	Bi et al 2015
gp78	Ubiquitination	Liu et al 2014
Cue5	Autophagy	Lu et al 2014
AUP1	Lipid droplet formation	Lohmann et al 2013
Cue1p	ERAD	Bagola et al 2013
FANCD2	Protein stability	Rego et al 2012
CUEDC2	Cell-cycle regulation	Gao et al 2011
TAB2	Cell signalling	Kishida et al 2005

1.5 Presenilin/γ-secretase Structure

As membrane bound proteins the structures of the presenilin proteins and the γ-secretase complex has been difficult to determine. However, in recent years these protein structures have begun to become clearer through a combination of mutagenesis studies, electron microscopy, NMR and x-ray crystallography [Lu et al 2014; Takagi Niidome *et al* 2015; Sobhanifar *et al* 2010; Li *et al* 2012]. Analysis of the presenilins primary sequence showed that presence of the PAL motif in the C-terminus of the proteins, which was shown to be required for complex formation and γ-secretase activity **(Figure 1.4)** [Tomita *et al* 2001; Wang *et al* 2005]. Using a method of cysteine substitution, allied with crosslinking the PAL domain of PS1 has been shown to be located near the active site of the protein [Tolia et al 2008]. NMR

analysis of the PS1 CTF has shown that of the 3 TMDs (relating to TMDs 7-9), only TMD 8 fully crosses the membrane. TMD 7 does not fully transverse the bilayer while TMD 9 is kinked such that it does not cross the bilayer but instead some of the helix lies perpendicular to the other TMDs [Sobhanifar *et al* 2010]. This study also showed that TMDs 7 and 9, which both play a role in the protease's active site, are water accessible. Other studies, using the substituted cysteine accessibility method (SCAM), have shown that hydrophilic loop 1 and the C-terminal of PS1 also play an important role in substrate binding in the gamma secretase complex [Takagi Niidome *et al* 2015]. While all of these studies have illuminated some of the finer details of presenilin structure until recently the overall structure of the presenilins has remained elusive.

There have been some significant advances in the use of x-ray crystallography to determine the structure of the presenilins in recent years. An archeal homolog of the presenilins called presenilin/SPP homolog (PSH) from *Methanoculleus marisnigri* was shown to have proteolytic activity [Li et al 2012]. PSH was subjected to a systemic program of mutagenesis that would promote protein crystallisation while retaining protease function. In the predicted PSH structural model the TMDs 1-6 form an outer ring which surrounds TMDs 7-9, with two potential sites of substrate entry being possible between either TMDs 6 and 9 or between TMDs 6 and 2. The overall three-dimensional shape of PSH was found to be unlike the structure of any known protein so the authors have named the structure the 'presenilin fold' [Li et al 2012]. Once the crystal structure of this presenilin homolog had been determined an *in silico* model of human PS1 was constructed. Using these models 12 different FAD mutations, that were present at conserved residues found in both PSH and human PS1, were analysed for their effect on protease activity. Two of the investigated FAD

mutations had no effect on protease activity and were found to be located away from the active site. Two of the other investigated residues were in direct contact with the proteolytic Asp162 residue of PSH and where mutation of this residue completely eliminated protease activity. Another group, which decreased proteolytic activity, were present near the proteolytic Asp residues of TMDs 6 and 7 [Li *et al* 2012]. This study provided great insight into both presenilin protein structure and γ-secretase activity, particularly in terms of the effects of presenilin FAD mutants, and could aid in the development of designed γ-secretase inhibitors and modulators.

While the structure of the γ-secretase complex has yet to be determined by determined by x-ray crystallography, electron microscopy has been used with increasingly effective results in elucidating the three-dimensional structure of the complex. Firstly, intact γ-secretase complexes were purified from Chinese hamster ovary (CHO) cells and analysed by cryo-electron-microscopy (cryo-EM) with a resolution of about 15 A° [Lazarov *et al* 2006]. The γ-secretase complex was found to ~120 A° in length with a pore of 20-40 A° in size that was thought to represent the substrate cleavage site. Using tagged Nct expressing in CHO cells, the γ-secretase complex was purified and analysed by STEM to achieve a resolution of 12 A° [Osenkowki *et al* 2009]. Their analysis showed that the γ-secretase complex had a molecular weight of ~230 kDa with a proposed stoichiometry of 1:1:1:1 for each subunit. The extracellular portion of the complex showed a greater degree of heterogeneity than the relatively smooth cytosolic portion. Additionally they showed that there was an opening on the cytosolic side that extended about half way into the structure and two large holes that could represent pores. Using a similar method, γ-secretase complexes purified from human HEK293T cells were analysed by

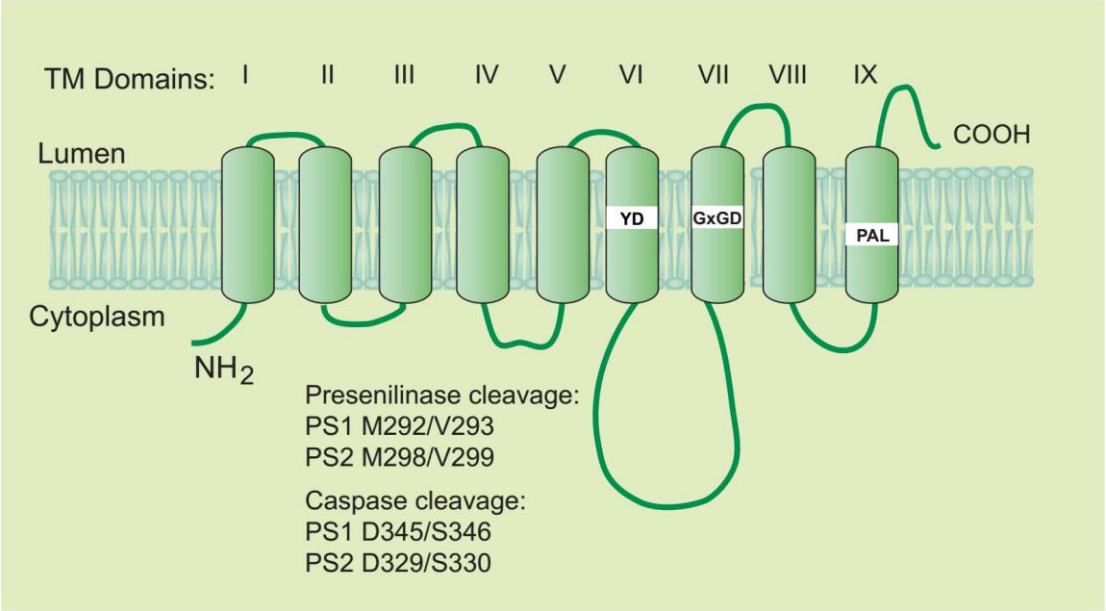


Figure 1.4 Schematic of the Structure of the Presenilin Proteins. The presenilins are membrane bound proteins that contain 9 transmembrane (TM) domains. The presenilins contain a large intracellular loop between TM domains 6 and 7 which contains the sites of presenilinase and caspase cleavage. The presenilins are aspartyl proteases where the protease active sites are found at the YD and GxGD sites in TM6 and TM7, respectively. This figure was taken from [Duggan and McCarthy 2016].

cryo-EM to obtain a resolution of 4.5 A° [Lu et al 2014]. In their model the whole γ-secretase complex has a horseshoe shape and a horizontal wedge shape with one end thicker than the other. Nct extended 40 A° above the membrane; Pen-2 binds to TMD 4 of PS1 while Nct and Aph-1 interacted with PS1 CTF. TMDs 6 and 7, which contain the catalytic Asp residues, and TMD 9 of PS1 all face towards the central hollow of the complex representing their importance towards the proteolytic activity of the complex. An *in silico* model of PS1 was made by conflating the date retrieved from the previously described NMR structure of PS1 CTF and the x-ray crystallography model of PSH [Kong et al 2015]. In this model the substrate gate for presenilin/gamma secretase is formed by the gap between TMDs 2 and 6 of presenilin. The computer model also showed that TMD 9 and its PAL motif is highly mobile and flexible, backing up its suggested role in regulating substrate recognition and cleavage.

The presenilin proteins have been suggested to act as passive calcium leak channels in the ER [Lu et al 2006]. Using the SCAM method residues within the TMDs 6, 7 and 9 were found to be required for PS1 calcium pore function with TMDs 7 and 9 seen to be most important [Nelson *et al* 2011]. In this study the residues within TMD 9 were found throughout the entire domain, while in TMD 7 they were found to be present around the catalytically essential Asp385 residue. Analysis of the PSH protein showed the existence of a 'hole' that extends through the membrane that is separate to the substrate cleavage site [Li et al 2012]. This hole is big enough to allow the passage of small ions through the protein, though the authors suggest that it is probably filled with lipid molecules *in vivo*. A recent paper showed that the PSH protein is able to function as an ion channel for Na^+ and K^+ ions but not Ca^{2+} ions [Kuo *et al* 2015]. This ion channel activity of PSH was removed when the

intracellular loop between TMDs 6 and 7, suggesting that this ion channel function of PSH is a function of the full length protein. These structural studies have added some weight to the controversial presenilin Ca^{2+} ER leak channel theory. The structure of PSH was only determined after the intracellular loop between TMDs 6 and 7 had been removed [Li *et al* 2012]. The structure of the presenilins has mostly been studied in terms of the γ-secretase complex, where most of the work has been focused on the topology of the intramembrane domains and the structure of the catalytic active site. As such, the structure of the intracellular loop domain of the presenilins, which is a site of many protein-protein interactions and post-translational modifications [McCarthy *et al* 2009], remains to be resolved.

1.6 Presenilins and Cell Signalling

In addition to their role in the regulated intramembrane proteolysis as part of the γ-secretase complex the presenilins are also known to be involved in a number of γ-secretase independent functions **(Table 1.3)**. The presenilins are involved in signalling pathways such as β–catenin [Zhang *et al* 1998], insulin signalling [Maesako *et al* 2011] and Akt signalling [Zhang *et al* 2008] in a γ-secretase independent manner. FAD mutants of PS1 have been shown to increase apoptosis by reducing Akt signalling [Weihl *et al* 1999]. Presenilin double knockout (PSDKO) cells have a much reduced level of Akt signalling which is rescued by γ-secretase redundant PS1 mutants suggesting that the presenilins have a γ-secretase independent role in regulating Akt signalling [Zhang *et al* 2008]. This reduction in Akt signalling always includes reductions in Erk signalling and is mediated by cell surface receptors such as Platelet-derived growth factor (PDGF) and the cadherins [Kang *et al* 2005]. Inhibition of the Akt downstream target GSK3β by insulin

treatment increases presenilin cell surface localisation and treatment with Akt inhibitors help reduce presenilin interactions with N-Cadherin and β-catenin suggesting that the presenilins play a role in Akt signalling [Maesako *et al* 2010].

Table 1.3 List of γ-secretase independent functions of the presenilin proteins

γ-secretase Independent Presenilin Functions
Regulation of beta-catenin/Wnt signalling
Regulation of calcium homeostasis
Regulation of apoptosis
Inflammation
Protein trafficking

The catenins are signalling protein that links transmembrane adhesion proteins, such as the γ-secretase substrate E-cadherin [Marambaud *et al* 2002], with intracellular signalling pathways and is involved in regulating transcription in a number of anti-apoptotic/survival pathways [Rosenbluh *et al* 2014]. The presenilins have been shown to be involved in β-catenin signalling in two ways **(Figure 1.5A)**. Firstly, cadherin proteins have been shown to be γ-secretase substrates causing the catenins to dissociate from the cell membrane [Marambaud *et al* 2002; Haas *et al* 2005] and secondly PS1 has been shown to interact with the catenins such as β-catenin [Murayama *et al* 1998], δ-catenin [Zhou *et al* 1997] and plakoglobin [Raurell *et al* 2006]. PS1 FAD mutant has been shown to alter β-catenin signalling thus inducing apoptosis in neuronal cells [Zhang *et al* 2008]. FAD PS1 mutants inhibit Wnt signalling by increasing β-catenin phosphorylation [Kawamura *et al* 1998]. The serine residues Ser353 and Ser357, GSK3β phosphorylation sites located within the

intracellular loop domain of PS1, have been shown to regulate the interaction between PS1 and β-catenin *in vivo* [Kirschenbaum *et al* 2001] and to increase β-catenin stability by reducing its ubiquitination and degradation [Prager *et al* 2007]. However, these mutations have no negative effects on cell survival, Wnt signalling or Aβ production in HEK293T cells. Similarly, the GSK3β mediated phosphorylation of residues Ser353 and Ser357 has been shown to reduce the association of PS1 and N-cadherin and β-catenin and a decrease in this association negatively affects Akt signalling [Uemura *et al* 2007]. PS1 is an unprimed substrate of GSK3β [Twomey and McCarthy 2006] and contains three consensus sites for GSK3β phosphorylation. Mutations at Ser397, located within another GSK3β binding site, increase the stability of the PS1 CTF; [Kirschenbaum *et al* 2001] this effect is also seen in cells treated with the GSK3β inhibitor lithium chloride. This increased stability is not repeated in the full-length protein or the NTF suggesting that GSK3β plays a role in targeting the CTF for degradation. The turnover of the PS1 binding protein δ-catenin is regulated by its GSK3β mediated phosphorylation, which induces its ubiquitination and proteosomal degradation [Oh *et al* 2009]. While in presenilin deficient cells an increase in Wnt signalling has been shown to be the result of sequestering of GSK3β to the endosome [Dobrowolski *et al* 2012]. PS1 has been shown to have both γ–secretase-dependent and -independent roles in β-catenin mediated transcription, where the PS1 holoprotein and the processed PS1 fragments have contrasting roles in modulating β-catenin/Tcf-4 gene transcription [Raurell *et al* 2008]. Additionally, defects in neurite growth observed in FAD murine cells can be reduced by inhibiton of β-catenin mediated gene transcription [Teo *et al* 2005]. Thus PS1 is heavily implicated in the regulation of β-catenin/Wnt signalling through both

γ-secretase dependent- and -independent functions. These roles are regulated through the post-translational modification of both PS1 and the catenin proteins.

1.7 Presenilins and Calcium Homeostasis

Another proposed ancestral role for presenilins relates to the formation of calcium leak channels and the regulation of intracellular calcium homeostasis **(Figure 1.5)** [Zhang *et al* 2010; Zhang *et al* 2015]. Disrupted calcium signalling has been reported to precede the classic AD pathologies, neurofibrillary tangles and Aβ plaques, and is proposed to contribute to neuronal cell death during AD [Bezprozvanny *et al* 2008]. Similarly, in numerous mouse models of AD increased basal levels of Ca^{2+} have been observed and presenilin FAD mutations have been observed to cause changes in Ca^{2+} levels [Zhang *et al* 2010; Jung *et al* 2011; Nelson *et al* 2010; Tu *et al* 2006]. There are several lines of evidence supporting a role for presenilins in the regulation of calcium homeostasis of intracellular stores, which appears to be independent of γ-secretase protease activities (**Figure 1.5B**). First, the presenilins interact with several proteins functionally involved in calcium signalling, including sorcin, calmodulin, calsenilin, calmyrin and calpain [Stabler *et al* 1999; Pack-Chung *et al* 2000; Shinozaki *et al* 1998]. It was next proposed that presenilin holoproteins act as passive Ca^{2+} channels in the ER and those PS FAD mutations alter channel conductance [Tu *et al* 2006]. In an elegant mutagenesis study it was subsequently demonstrated that the hydrophilic catalytic cavity of PS1 facilitates the formation of a calcium leak conductance pore [Nelson *et al* 2011]. In parallel, the presenilins have been shown to regulate Ca^{2+} levels through interactions with and activation of Ca^{2+} channels such the sarco/ER Ca^{2+}-ATPase (SERCA) pump [Green *et al* 2008], the inositol triphosphate receptor (InsP3R) [Cheung *et al* 2008], and the Ryanodine receptor (RyR) [Rybalchenko *et al* 2008; Hayrapetyan *et al* 2008]. More recently the

presenilins have also been reported to modulate phosphatidylinositol-4,5-bisphosphate (PIP2)-mediated regulation of the transition receptor potential of melastatin related 7 (TRPM7) [Oh *et al* 2012]. Finally, the presenilins have also been proposed to increase the number of contact sites between the ER and mitochondria, thereby facilitating movement of Ca^{2+} from the ER to the mitochondria [Zampese *et al* 2011].

PS1 FAD mutations and an Alzheimer's triple transgenic mouse model (3xTg) show increased Ca^{2+} signalling while use of a dominant negative allele of InsP3R has been shown to reduce AD-like symptoms in 3xTg mice, suggesting the importance of Ca^{2+} in AD progression [Shilling *et al* 2014]. Furthermore, presenilin-deficient MEFs show a decrease in ER Ca^{2+} concentration, which may be as a result of the presenilins regulating InsP3R levels [Kasri *et al* 2006] or SERCA pump levels leading to increased ER [Ca^{2+}] [Green *et al* 2008], which in both cases normal Ca^{2+} levels can be rescued by overexpression of presenilin holoproteins. Mouse models expressing PS1 FAD mutants show constitutive activation of cAMP response element binding (CREB) gene expression, which is eliminated by inhibition of InsP3R [Muller *et al* 2011]. PS1 FAD M146V mutant knock-in hippocampal cells showed reduced synaptic plasticity and defects in long-term potentiation (LTP), which can be rescued by inhibition of the RyR [Zhang *et al* 2015]. Overexpression of both PS1 and PS1 FAD mutants have been shown to increase dendritic density in murine cortical neurons in a Ca^{2+} signalling-mediated and γ-secretase-independent manner [Jung *et al* 2011] and PS1 FAD mutants have been shown to enhance [Ca^{2+}] in human lymphoblasts [Nelson *et al* 2010]. PS1 FAD mutations have been shown to both decrease and increase PS1 mediated ER Ca^{2+} release [Nelson *et al* 2010; Tu *et al* 2006]. Conditional knockout of presenilins or expression of the PS1 FAD M146V

mutant in mouse hippocampal cells has been shown to increase ER Ca^{2+} levels and enhance susceptibility to induced Ca^{2+} release [Zhang *et al* 2010]. These cells also showed an increase in ER RyR levels, which the authors suggest, maybe a result of the decreased presenilin leak channel function, leading to the increased ER Ca^{2+} levels and thus a need for an increase in ER Ca^{2+} channel expression.

Down-regulation of PS1 via inhibition of JNK or P53 has been shown to decrease ER Ca^{2+} leakage without altering InsP3R expression [Das *et al* 2012]. TRAF6 has been shown to stabilize full length PS1 and both presenilin-deficient and TRAF6-deficient MEFs have been shown to alter ER Ca^{2+} release [Yan *et al* 2013]. Consistent with mammalian studies and supportive of an evolutionary conserved function, X-ray crystallography studies of a presenilin archaeal homologue PSH have shown the presence of a hole that passes through the protein formed from TMD2, TMD3, TMD5 and TMD7, separate to the catalytic core of the enzyme, that is large enough to allow small ions to pass through. The authors suggest that rather than being a second channel this hole is probably filled by lipid molecules *in vivo* [Li *et al* 2012]. Despite all of these studies, some controversies have been raised by reports of the direct measurement of Ca^{2+} ER release from primary neurons, fibroblasts, B-cells and hippocampal neurons [Shilling *et al* 2012; Wu *et al* 2013], which showed no difference in ER Ca^{2+} dynamics between cells expressing PS1 WT and PS1 FAD mutants and that any change in ER [Ca^{2+}] in presenilin-deficient cells was not due to increased ER filling [Shilling *et al* 2012]. Therefore, while the presenilins have been shown to be important in regulating intracellular Ca^{2+} via several proposed mechanisms, as it stands the Ca^{2+} leak channel function theory of the presenilins remains controversial.

1.8 Presenilins and Apoptosis

Apoptosis is the process where external or internal cellular signals lead to a program of controlled cell death [Orrenius *et al* 2015]. In the extrinsic pathway ligands, such as tumour necrosis factor α (TNFα) or FasL, bind to cell surface death receptors (DRs), such as the TNFα receptor type 1 (TNFR1), to initiate a pro-apoptotic cell signalling pathway [Gonzalvez and Ashkenazi 2010]. The DRs contain death domains which can interact with death domain containing adaptor proteins, such as TNFR-associated death domain (TRADD), leading to the formation of a death-inducing signalling complex (DISC) that activates the initiator caspase caspase-8. The activation of these initiator caspases leads to the cleavage and activation of the proteases caspase-3, -6 and -7, which ultimately carry out the apoptotic signal within the cell [Gonzalvez and Ashkenazi 2010]. In the intrinsic pathway internal signals such as reactive oxygen species, nutrient depletion or DNA damage lead to the activation of pro-apoptotic Bcl-2 family proteins, such as Bax, leading to the release of cytochrome *c* from the mitochondria and activation of the apoptotic initiator caspase-9 [Wu and Bratton 2013]. In AD abnormal levels of apoptotic associated proteins such as caspases-8 and -9, Bax and Bcl-2 proteins have been detected within the brain tissue of AD patients and accumulation of amyloidogenic Aβ40/42 peptides leads to the induction of apoptosis in cultured neurons [Calissano *et al* 2009]. The presenilins were first linked to apoptosis when cells expressing PS1 FAD mutants were shown to have increased susceptibility to apoptotic stimuli [Guo *et al* 1996; Yang *et al* 2008]. Both presenilin proteins are cleaved by caspase-3 during apoptosis to form alternative NTF and CTF fragments for each protein [Kim *et al* 1997]. Phosphorylation of residues S327 and S330 protects PS2 from caspase cleavage

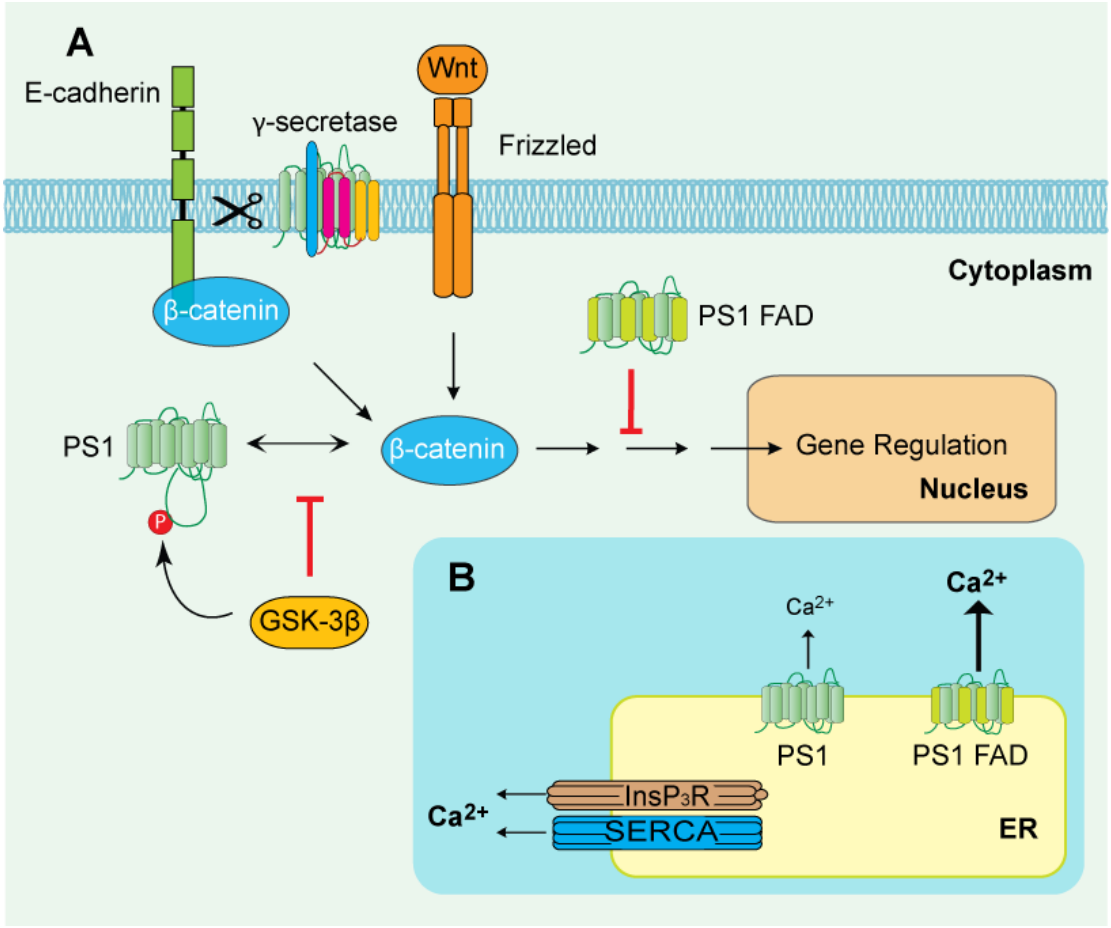


Figure 1.5 Presenilin Involvement in β-Catenin Signalling and Calcium Homeostasis. (A) The γ-secretase cleavage of E-cadherin causes the release β-catenin from the plasma membrane. PS1 interacts with β-catenin, a signalling molecule that is involved in Wnt signalling and gene regulation, while PS1 FAD mutants have been shown to inhibit these signalling events. The interaction between PS1 and β-catenin is also inhibited by the phosphorylation of PS1 by GSK3β. (B) PS1 is known to be involved in regulating calcium homeostasis in the ER in a number of ways. Firstly, PS1 has been shown to interact with calcium channels InsP3R and SERCA. Secondly, PS1 has been hypothesised to act as a calcium leak channel within the ER. Additionally, PS1 FAD mutants have been shown to increase calcium release from the ER. This figure was taken from [Duggan and McCarthy 2016].

[Walter *et al* 1999]. Mouse B-cells deficient in PS1 or PS2 show an increased susceptibility to LPS induced apoptosis [Yagi *et al* 2008], while increased levels of neurodegeneration and apoptosis are observed in the brains of an Alzheimer's PS/APP mouse model [Yang *et al* 2008]. Expression of the PS1 FAD mutants ΔE9 and L250S lead to an increased susceptibility to apoptosis in neuroblastoma cells [Tanii *et al* 2000]. Expression of PS1 and PS2 in HeLa cells has been shown to cause cell cycle arrest and apoptosis and the expression of the FAD mutants of either presenilin protein leads to an increased level of apoptosis [Janicki *et al* 1997; Janicki *et al* 1999; Janicki *et al* 2000]. While both presenilins are involved in apoptosis they can sometimes play opposing roles in regulating apoptotic pathways. Overexpression of PS1 is able to reduce p53 mediated apoptosis in the LTR6 mouse leukaemia cell line [Amson *et al* 2000], whereas PS2 expression increases apoptosis via p53 where it increases Bax expression and reduces Bcl-2 expression [Alves da Costa *et al* 2003]. PS1 has been shown to regulate apoptosis in both γ-secretase-dependent and –independent ways via c-FLIP and the presenilin-1 associated protein (PSAP), respectively **(Figure 1.6)** [Zeng *et al* 2015]. PSAP is predominantly expressed within the brain and interacts with the PDZ interacting motif at the C-terminal of PS1 [Xu *et al* 1999] PSAP has been shown to regulate apoptosis via caspase signalling and by controlling the release of cytochrome C from the mitochondrial [Xu *et al* 2002]. Silencing of the pro-apoptotic Bcl-2 family Bax protein repressed this γ-secretase independent apoptotic pathway [Zeng *et al* 2015] suggesting that the interaction between PS1 and PSAP plays a γ-secretase independent role in regulating the mitochondrial apoptotic pathway. PS1 directly interacts with the Bcl-2 leading to alterations in apoptosis [Alberici *et al* 1999]. This presenilin mediated apoptosis is modulated by the FKBP38 protein, as FKBP38 knockout cells alter Bcl-2 cell

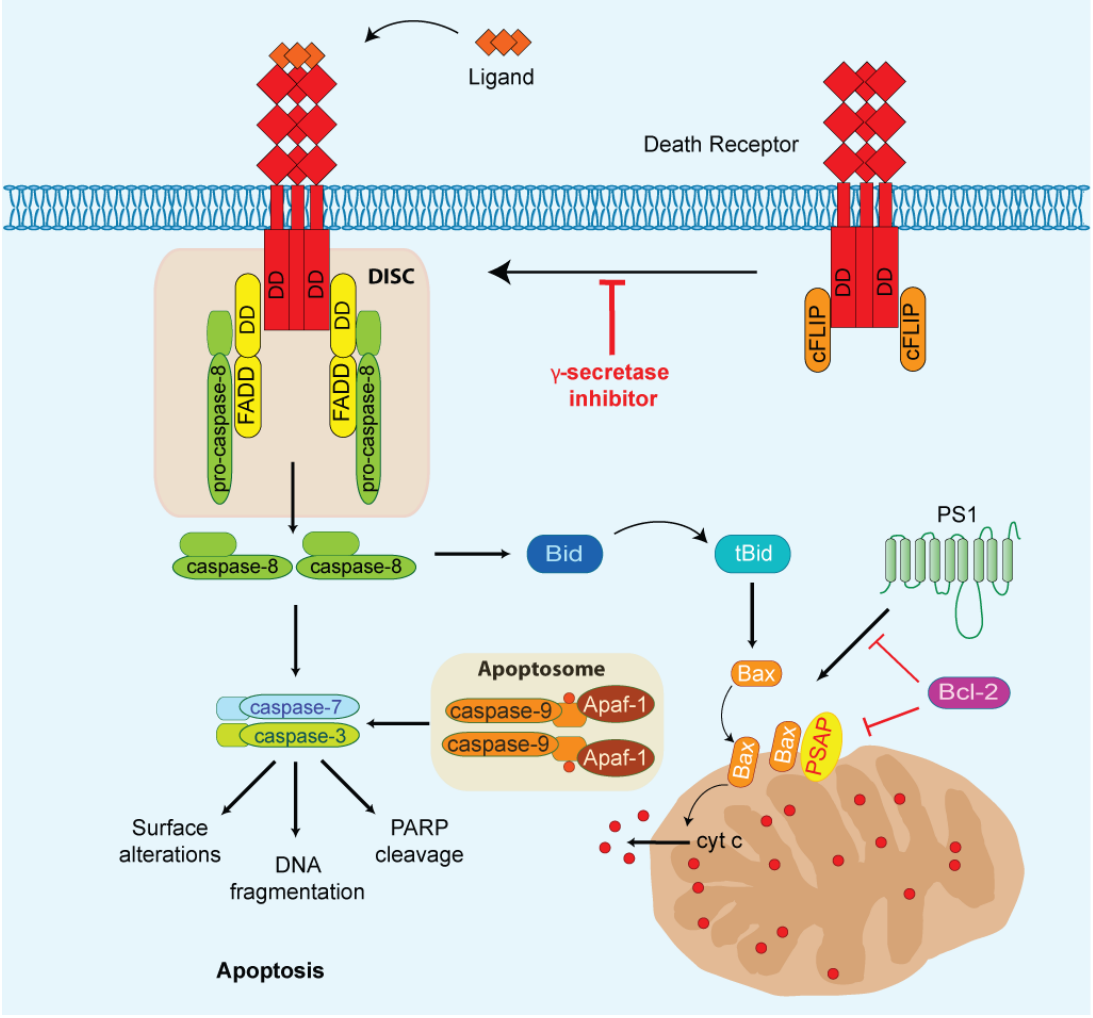


Figure 1.6 The presenilins play a γ-secretase-dependent and –independent role in the regulation of apoptosis. This schematic shows a representation of the extrinsic apoptotic pathway involving the induction of an apoptotic signal after the ligand binding to a death receptor leading to the downstream activation of the caspases and the initiation of apoptosis. Treatment with γ-secretase inhibitors can inhibit this pathway. PS1 promotes the interaction of PSAP and Bax via a γ-secretase independent pathway leading to mitochondrial membrane breakdown and release of cytochrome c into the cytoplasm and the activation of caspase 9 leading to the induction of apoptosis. This figure was taken from [Duggan and McCarthy 2016].

localisation protecting them from presenilin mediated apoptosis [Wang *et al* 2005]. Therefore, both presenilin proteins can be seen to play distinct roles in regulating apoptosis though due to the involvement of the presenilins in a variety of other cellular processes, the exact role played by the presenilins in apoptosis remains unclear.

1.9 Presenilins and Inflammation

Aβ deposits are known to induce inflammation within the brain, where both components of the complement system and the pro-inflammatory cytokine IL-1 are raised within the brains of AD patients [McGeer and McGeer 2013]. Alzheimer's disease cases associated with FAD PS1 mutations are known to contain plaques that are enriched with microglial cells involved in the inflammatory response [Shepherd *et al* 2005]. Inhibition of γ-secretase activity leads to delayed CD8 T-cell development [Doerfler *et al* 2001] while conditional presenilin knockout mice show a deficit in CD4 thymocytes and a reduction in T-cell receptor signalling [Laky and Fowlkes 2007]. Mice with conditional knockout of presenilins have been shown to have alterations in their inflammatory response in both the neuronal system and the periphery [Jiang *et al* 2009]. There is an increase in TLR4 mediated cytokine expression in mice expressing the APP Swedish mutation and the PS1 ΔE9 FAD mutant [Jin *et al* 2008]. They have also been shown to have an autoimmune phenotype that is similar to systemic lupus erythematosus [Tournoy *et al* 2004]. These alterations included increased activation of glial cells and astrocytes, increased activation of the complement system and alterations in expression of pro-inflammatory cytokines.

Mice overexpressing the PS1 M146V FAD mutation showed a significantly larger increase in TNF and IL-1β transcription and an increase in microglial activity following stimulation with LPS [Lee *et al* 2002]. LPS treatment is also known to downregulate PS2 expression in mice [Saban *et al* 2002] and bone marrow derived macrophages [Agrawal *et al* 2015]. By contrast PS1 expression increases in the brains of mice treated with LPS [Nadler *et al* 2008]. Presenilin deficient lymphocytes have been shown to have a reduced capability to differentiate after LPS treatment whilst also showing a reduction in signal transduction [Yagi *et al* 2008]. Presenilin deficient mice have also shown an upregulation in genes involved in the immune system such as the complement gene C1q and the glial marker GFAP [Beglopoulos *et al* 2004]. PS1 and PS2 have different effects in the inflammatory response where PS2KO cells show different responses to LPS stimulation compared to WT or PS1KO cells [Agrawal *et al* 2015]. This change in cytokine response to LPS in PS2KO cells is not affected by γ-secretase inhibitors suggesting that this PS2 function in the inflammatory response is γ-secretase independent. PS2 has also been shown to upregulate the expression of the immune system modulating miRNA miR146 in microglial cells and this decrease in miR146 leads to increases NFκB signalling [Jayadev *et al* 2013]. This suggests that the presenilin proteins may play conflicting roles in the regulation of inflammation though this process remains unclear at present.

1.10 Presenilins in Protein Trafficking and Degradation

A plant homologue of presenilin (PpPS) from *Physcomitrella patens* has been shown to be involved in regulating organelle movement and cytoskeleton remodelling within plant cells [Khandelwal *et al* 2007]. The PpPS protein does not show any protease activity and PpPS with mutations at the typical catalytic aspartate sites were

able to rescue defects in protein trafficking in PpPS null cells. This process was also rescued by human PS suggesting that this trafficking function is both γ-secretase independent and conserved through evolutionary time. Similarly in the amoeba *Dictyostelium discoideum*, the proteolytically active homologs of the presenilins have been shown to be required for both differentiation and for bacterial phagocytosis [McMains *et al* 2010]. The *Dictyostelium* presenilins have also been shown to play a γ-secretase independent role in development [Ludtmann *et al* 2014]. In mammalian cells the presenilins and γ-secretase were first shown to play an important role in protein trafficking within the cell and in regulating protein transport within the axons of neurons [Naruse *et al* 1998]. Presenilins have been shown to regulate axonal transport via interactions with GSK3β thus influencing kinesin-1 and dynein activity [Dolma *et al* 2014], and reduction in γ-secretase activity accelerates APP axonal transport via kinesin-1 and dynamin [Gunawardena *et al* 2013].

Axons of mice expressing the PS1ΔE9 mutant also show reduced kinesin-1 activity and reduced fast axonal transport of type 1 transmembrane receptors such as APP and Trk [Lazarov *et al* 2007]. Presenilin conditional knockout (PScDKO) mice, where the presenilin genes are selectively knocked out in neuronal cells, show a reduction of the NMDAR regulating protein drebrin A at the synapse [Lee *et al* 2012]. Overexpression of the PS1 FAD mutant M146L alters the distribution filamin, a protein that is involved in regulating neuronal migration, within the cell from the cell periphery to the cytoplasm; this alteration is not changed by treatment with a γ-secretase inhibitor (GSI) suggesting that PS1 regulates filamin localisation in a γ-secretase independent manner [Lu *et al* 2010]. These studies demonstrate that the presenilins play an important role in maintaining axonal transport within neurons and in regulating neuronal migration.

The presenilins regulate trafficking of proteins to the cell surface through both γ-secretase dependent and independent functions. A number of sorting receptors, such as Sortilin, SorCS1b and SorLA, have been shown to be γ-secretase substrates [Nyborg *et al* 2006]. The presenilins are involved in regulating the trafficking of APP in neurons [Gandy *et al* 2007] and deletion of PS1 leads to an increased level of APP full length and CTF at the cell surface [Leem *et al* 2002]. MEFs expressing FAD PS1 mutants have abnormal APP trafficking leading to irregular APP glycosylation [Piccini *et al* 2004], and have also been shown to impair fast axonal transport (FAT) leading to changes in APP trafficking [Lazarov *et al* 2007]. While inhibition of γ-secretase can have a negative effect on trafficking [Weissmiller *et al* 2015; Zhang *et al* 2006], the presenilins can also affect trafficking in γ-secretase independent ways. PS1 trafficking to the cell surface is reduced and PS1 ER localisation is increased by overexpression of calsenilin and this in turn reduces the cleavage of N-Cadherin [Jang *et al* 2011; Jang *et al* 2011]. Presenilin deficient neurons have been shown to have a reduced level of trafficking of TrkB and EphB receptors to the plasma membrane [Naruse *et al* 1998; Barthet *et al* 2013] and that the neuro-protective qualities of the EphB ligand efnB are abrogated in presenilin deficient neurons. This neuroprotection is maintained in wild type cells treated with γ-secretase inhibitors suggesting that this is a γ-secretase independent function of the presenilins [Barthet *et al* 2013]. By contrast, PSDKO MEFs showed an increase in integrin β1 trafficking to the cell surface and an increase in integrin β1 post-translational modification and maturation [Zou *et al* 2008]. The γ-secretase interacting protein CD147 translocation to the cell surface is reduced in PS2 deficient cells [Nahalkova *et al* 2010]. These results suggest that the presenilins play

a role in chaperoning proteins through the ER to the Golgi which is independent of presenilin's γ-secretase function.

Presenilins have been shown to regulate the turnover of a number of proteins. The PS1 CTF has been shown to mediate EGFR turnover via the endosomal/lysosomal system and as such regulates EGFR signalling [Repetto *et al* 2007]. PS1 regulates EGFR turnover and signalling by down-regulating the E3 ligase Fbw7 via Fbw7 interactions with the PS1 intracellular loop [Rocher-Ros *et al* 2010]. The neuronal cell adhesion protein telencephalin has been shown to interact with PS1 and been shown to increase in half-life and accumulate in autophagic vacuoles in PS deficient neurons [Esselens *et al* 2004]. There is an increased level of autophagic vacuoles (AVs) detected in neurons of human AD patients and in the neurons of AD mice models [Yu *et al* 2005]. Mice containing the FAD APPswe and PS1 ΔE9 mutations have shown decreased levels of the autophagy regulator mTOR and increased levels of AVs and increased levels of inflammation [Francois *et al* 2015]. Deletion of the presenilins, but not inhibition of γ-secretase activity, has been shown to cause a reduction in autophagy in both MEFs and in neuroblastoma cells [Neely *et al* 2011]. Treatment with the autophagy inducer rapamycin could not rescue autophagy in these presenilin deficient cell lines. Ubiquilin, a presenilin interacting protein, has been shown to regulate autophagy and thus rescue HeLa cells from cell death mediated by the PS2 N141I FAD mutant [Rothenburg *et al* 2010]. From this it is clear that the presenilins play an evolutionary conserved role in protein trafficking and they demonstrate both γ-secretase-dependent and -independent roles in protein trafficking and turnover.

1.11 Therapeutic Implications of γ-Secretase-Independent Functions of Presenilins

Due to their role as the enzymatic core of the γ-secretase complex the presenilin proteins and the prevalence of presenilin mutations in the development of FAD, the presenilins have been an obvious drug target for many years. Therefore GSIs have principally been designed as a means of reducing the formation of toxic Aβ species. GSIs were first designed as transition-state peptide analogues of γ-secretase substrates before the development of non-transition state γ-secretase inhibitors which target the γ-secretase active site such as N-[N-(3,5-difluorophenacetyl)-1-alanyl]-s-phenylglycine t-butyl ester (DAPT) or the structurally related Compound E [Wolfe 2008]. The large number of γ-secretase substrates and the central importance of substrates such as Notch to cell differentiation has led to the search for γ-secretase modulators (GSMs), which would preferentially target toxic Aβ production without affecting other γ-secretase substrate cleavage [Weissmiller *et al* 2015; Liu *et al* 2014]. There has also been some success in decreasing Aβ formation through modulating lipid raft formation and thus decreasing γ-secretase activity [Kang *et al* 2013]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen have been used as GSMs to shift the amyloid production from toxic Aβ40/42 species towards the longer Aβ peptides, while some more potent GSMs shift the production down towards shorter Aβ37/38 species [Golde *et al* 2013]. Targeting of γ-secretase activity is also a target in cancer treatment. The *Notch1* gene is mutated in up to 50% of T-cell acute lymphoblastic leukemia/lymphoma while Notch 1 and 4 and the Notch ligands Delta and Jagged, which are also γ-secretase substrates, are highly expressed in endothelial cells [Takebe *et al* 2015]. Inhibition of Notch signalling

with the GSI MRK-003 lead to a decrease in cell growth and an increase in apoptosis in a number of glioblastoma cell lines [Chen *et al* 2010].

Alterations in presenilin mediated cell signalling have been shown by inhibiting γ-secretase and by inhibiting other cell pathways. Treatment with a number of GSIs has been shown to induce caspase-3 mediated apoptosis in both normal and presenilin-deficient cells which suggests that treatment with GSIs may have off target effects other than inhibiting γ-secretase activity [Alves da Costa *et al* 2004]. On the other hand presenilin/ γ-secretase activity can be affected by targeting other signalling pathways, for example astroglial cells treated with cadmium induces the γ-secretase cleavage of N-cadherin and this cleavage can be reduced by treatment with MEK1/2 inhibitors [Jo *et al* 2013]. Treatment with γ-secretase inhibitors has been shown to slow proliferation in multiple myeloma cells by lowering Notch signalling and stopping G0/G1 changeover [Hu *et al* 2013]. A combinational therapy of the GSI DAPT and the ERK inhibitor PD98059 has been shown to be effective in inducing apoptosis in gastric cancer cell lines [Yao *et al* 2013]. This effect is a result of DAPT inhibiting Notch leading to increased β-catenin signalling. Instead of a GSI the interaction between the presenilins and β-catenin can be modulated by treatment with LiCl, an inhibitor of GSK3β [Murayama *et al* 1998]. On the other hand, the interaction between PS1 and β-catenin and β-catenin signalling are not affected by treatment by another GSI compound E [Meredith *et al* 2002], suggesting that GSMs and GSIs may have different effects on the same signalling pathway. Treatment with compound E has no effect on cytoplasmic Ca^{2+} level in cells treated with the SERCA inhibitor thapsigargin [Das *et al* 2012]. However, treatment with DAPT has been shown to lead to an increase in InsP3R expression [Kasri *et al* 2006] suggesting that

different GSIs may have differing effects on modulating presenilin dependent Ca^{2+} signalling.

Non-steroidal anti-inflammatory drugs (NSAID) have been used as a part of Alzheimer's treatment for many years. Treatment with the NSAID ibuprofen has been shown to reduce expression of pro-inflammatory cytokines and prevent dental tissue damage in presenilin deficient mice [Su *et al* 2013]. Additionally treatment with intravenous immunoglobulin (IVIg) has been shown to reduce inflammation and reduce Aβ levels at a slower rate but for a long lasting period than treatment with an anti-APP antibody in APP/PS1 mice [Sudduth *et al* 2013]. Since the presenilin proteins, especially PS2, have been shown to have a γ-secretase independent role in regulating the immune system [Agrawal *et al* 2015] this could represent a novel drug target for inflammatory response in Alzheimer's disease. Similarly given the autoimmune diseases-like phenotype present in some PS cDKO mice [Tournoy *et al* 2004] the presenilin proteins could represent a potential target for the treatment for autoimmune diseases.

1.12 Concluding remarks

The presenilins are essential parts of the γ-secretase enzyme complex and as such control the regulated intramembrane proteolysis of over 100 transmembrane proteins including APP and Notch. These cleavage events play important roles in cellular differentiation, gene transcription and disease progression. In addition to its γ-secretase-dependent functions the presenilins have a number of γ-secretase independent roles in cell signalling, calcium homeostasis and protein trafficking. These γ-secretase independent roles allow the presenilins another means to regulate cell signalling and gene transcription through pathways such as the β-catenin or Akt pathways and also to affect apoptosis and the inflammatory response. As such these γ-secretase independent functions of the presenilins represent an additional consideration when targeting the presenilins for drug treatment. On the other hand these γ-secretase independent functions of presenilins also represent a potential source of drug targeting for modulating diverse cellular systems. Ubiquitination is a post-translational modification that is important in controlling protein turnover and diverse cell signalling pathways. The ubiquitination status of a protein can be 'read' by a number of ubiquitin binding domains, which can then transmit this signal on to other proteins to regulate diverse cellular processes. The presenilins undergo a number of post-translational modifications, including ubiquitination by SEL-10/Fbw7 and TRAF6, which can affect both γ-secretase dependent and independent functions of the proteins. In our studies we have identified a previously undescribed putative ubiquitin binding CUE domain in PS1 which is potentially important in deciphering the role played by ubiquitin in the regulation of PS1 functions.

The aims of this thesis are:

1. To functionally characterise the ubiquitin binding CUE domains of presenilins.

2. To investigate whether the presenilin CUE domains are involved in γ-secretase-dependent or –independent function(s) of the presenilins.

3. To develop and perform a mass spectrometry-based protein binding assay to identify ubiquitinated proteins that interact with the PS1 CUE domain and thereby elucidate the function of the PS1 CUE domain.

2 Materials and Methods

2.1 Chemicals and Reagents

All salts and reagents were purchased from Sigma-Aldrich (Dublin, Ireland) unless otherwise stated. Protein G-Sepharose beads were purchased from Invitrogen (Paisley, Scotland). Turbofect™ was obtained from Thermo Fisher Scientific via Medical Supply Company, Dublin, Ireland. Complete protease inhibitors were purchased from Roche (Boehringer-Mannheim, Indianapolis, USA). His-tagged recombinant human K48- and K63-linked polyubiquitin (chains of mixed length, 2-7 ubiquitin molecules) were purchased from Boston Biochem (Boston, USA). Protein molecular weight marker was obtained from Biorad Laboratories (Munich, Germany). ECL chemiluminescent kit was purchased from GE Healthcare (Buckinghamshire, UK). Phorbol 12-myristate 13-acetate (PMA) was obtained from Calbiochem (Nottingham, UK) Protran Nitrocellulose membrane was obtained from Perkin Elmer. Bicinchoninic acid (BCA) protein concentration reagents were purchased from Pierce Biotechnology (Rockford, Illinois).

2.2 Plasmid Sources

HA-P62 was a gift from Prof Jorge Moscat (Sanford Burnham Medical Research Institute, California, USA). pcDNa3.1-IL-1R1, PRK5-RIP1 and the FLAG-tagged PRK5-TRAF2 and FLAG-tagged PRK5-TRAF6 constructs were a gift from Dr Vishva Dixit (Genentech, California, USA).pCAX-FLAG-APPCT100 was a gift from Scios Inc (California, USA). pCS2+deltaEMV-6mt/NEXT was a gift from Raphael Kopan (Washington University). HA-Ub WT, HA-Ub $K48_{only}$, HA-UB $K63_{only}$ and HA-Ub K63R were a gift of Dr Ruaidhri Carmody (University of Glasgow). pKS-rat $p75^{NTR}$ was a gift of Prof Eric Shooter (Stanford University

School of Medicine, California, USA). pcDNA3-PS1 and pcDNA3-PS2 were previously made by members of the lab [Ciara Twomey]. The pGex-6P1 vector was purchased from GE Healthcare (Buckinghamshire, UK).

2.3 Molecular Biology Reagents

Primers were obtained from Sigma-Aldrich (Dublin, Ireland) or Integrated DNA Technology (Leuvan, Belgium). KOD Polymerase Kit and KOD Hot Start DNA Polymerase Kit was obtained from Calbiochem (Nottingham, UK). All restriction enzymes and T4 Ligase were purchased from New England Biolabs (Ipswich, USA). Gel extraction kit (catalog number 28104) and PCR purification kit (catalog number 28704) were obtained from Qiagen Ltd (West Sussex, UK). PureYield(TM) Plasmid Midiprep System kits were purchased from Promega through Millipore (Southampton, UK).

2.4 Antibodies

The 614.1 anti-PS1 NTF antibody was generated by Scios Inc. and has been previously described [Kirschenbaum *et al* 2001]. All other antibodies used in studies were obtained from commercial sources. Anti-Rab11 and anti-RIP1 antibodies were obtained from BD Bioscience. Anti-cleaved Notch (Val 1744) and anti-PS2 antibodies were purchased from Cell Signaling. Antibodies against PS1 CTF (MAB5232) and PS1 NTF (MAB1563) were obtained from Millipore; anti-HA antibody was obtained from Covance. Anti-mouse HRP and anti-rabbit HRP secondary antibodies were purchased from Dako Cytomation. IRDye 800CW anti-mouse and IRDye 800CW anti-rabbit secondary antibodies were purchased from Licor. The $P75^{NTR}$ antibody came from Promega. The IL-1R1 (C20), P4D1 anti-

ubiquitin and anti-TRAF2 antibodies were purchased from Santa Cruz Technology. The anti-actin, anti-FLAG and anti APP C-terminus antibodies were purchased from Sigma-Aldrich.

2.5 Chemical Competent Cell preparation of *E. coli* cells

DH5-α or BL21 *E. coli* cells were streaked on a Luria broth (LB) agar plate and grown overnight at 37°C. From this plate a single colony was picked and grown overnight in 5 ml of LB broth at 37°C. For BL21 cells, the LB agar and the LB broth was supplemented with 25 μg/ml chloramphenicol. The following day, 3 ml of the 5 ml DH5-α or BL21 cell cultures were added to 100 ml of LB broth and grown for 2-3 hours until an OD_{595} reading of 0.6-1.0 was reached. The culture was then centrifuged at 3000 rpm for 15 mins at 4°C. The resulting pellet was resuspended in one third volume of pre-chilled 0.1 M $MgSO_4$. This mixture was again centrifuged at 3000 rpm for 15 mins at 4°C and the resulting pellet was resuspended in 0.1 M $CaCl_2$ at $1/25^{th}$ of the volume of the original bacterial culture. This mixture was aliquoted, frozen on dry ice and stored at -80°C.

2.6 Bacterial Transformation of Competent Cells

The chemical competent DH5-α or BL21 *E. coli* cells were allowed to thaw on ice 15-20 mins prior to transformation. For plasmid DNA, 100 ng of the vector were incubated with 100 μl of competent cell suspension for 20 mins on ice, and then heat shocked at 42°C for 60 seconds. The cells were then cooled for 5 mins on ice before 900 μl of LB broth was added. The culture was then incubated for 60 mins whilst shaking at 37°C. 100 μl of this culture was then plated on LB-agar plates containing the appropriate antibiotic (50 μg/ml ampicillin or kanamycin; 25 μg/ml chloramphenicol) overnight at 37°C. For transformation of ligation or mutagenesis

reactions 5-20 µl of the reaction mix was incubated with 100 µl of competent cells and then were treated as described above.

2.7 Cloning and Mutagenesis

Site directed mutagenesis was carried out using KOD Hot Start as per the manufacturer's protocol and the primer pairs listed in Table 2.1. Primers for site-directed mutagenesis studies were obtained from Sigma-Aldrich (Dublin, Ireland) or Integrated DNA Technology (Leuvan, Belgium). Following successful amplification, the PCR reaction was purified using a PCR purification kit and then digested with the Dpn1 restriction enzyme to remove the methylated parental supercoiled dsDNA. The purified Dpn1 treated DNA mix was subsequently heat inactivated and 5 µl of mix was transformed into DH5-α cells as described above. Mutagenesis was confirmed via DNA sequencing (GATC Biotech, Konstanz, Germany) and sequence alignment using Vector NTI (Invitrogen).

Table 2.1 Paired Primers used in Cloning and Site-directed Mutagenesis

Mutation	Primer Sequence
P62 F406V	Forward 5' CCATGGGCGTCTCTGATGAAGGC 3' Reverse 5' GCCTTCATCAGAGACGCCCATGG 3'
PS1 ΔCUE	Forward 5' GATTTAGTGGCTTATAATGCAGAAAGCACAGAA 3' Reverse 5' TTCTGCATTATAAGCCACTAAATCATATACTGA 3' Forward 5' CGGGGTACCGCCATGACAGAGTTACCTGCACCGTTGTCC 3' Reverse 5' CCGGAATTCCTAGATATAAAATTGATGGAATGCTAATTG 3'
PS1 F283A/P284A	Forward 5' GAGAAATGAAACGCTTGCTGCAGCTCTCATTTACTCC 3'

	Reverse 5’ GGAGTAAATGAGAGCTGCAGCAAGCGTTTCATTTCTC 3’
PS1 V309A/S310A	Forward 5’ GGAAGCTCAAAGGAGAGCAGCCAAAAATTCCAAG 3’ Reverse 5’ CTTGGAATTTTTGGCTGCTCTCCTTTGAGCTTCC 3’
PS1 S310E	Forward 5’ GCTCAAAGGAGAGTAGAGAAAAATTCCAAGTATAATGC 3’ Reverse 5’ GCATTATACTTGGAATTTTTCTCTACTCTCCTTTGAGC 3’
PS1 Loop	Forward 5’ CGGGGATCCGCCGTTTTGTGTCCGAAAGGT 3’ Reverse 5’ CCGGAATTCCTATTTTACTCCCCTTTCCTC 3’
PS2 F289A/P290A	Forward 5’ GAAATGAGCCCATAGCCGCTGCCCTGATATAC 3’ Reverse 5’ GTATATCAGGGCAGCGGCTATGGGCTCATTTC 3’
PS2 L315A/Q316A	Forward 5’ CTCAGGGTGCCGCCGCGCTCCCCTACGACCC 3’ Reverse 5’ GGGTCGTAGGGGAGCGCGGCGGCACCCTGAG 3’

2.8 Construction of GST-tagged Expression Vectors

In order to generate a GST-tagged PS1 loop domain expression vector, the PS1 Loop primers (Table 2.1) were used to amplify the intracellular loop domain of human PS1 (nucleotides 792-1140 of the coding sequence, corresponding to amino acids 265-380,). The PCR products were digested with BamHI and EcoRI and then purified using the QiaQuick PCR purification kit. The pGex-6P1 GST expression vector was digested with BamHI and EcoRI, treated with alkaline phosphatase and gel purified using the QiaQuick gel extraction kit. The PCR products were ligated into the pGex-6P1 vector using the T4 ligase kit and the resulting mix was transformed into DH5-α cells as described above. DNA sequencing (GATC Biotech, Konstanz, Germany) and sequence alignment using Vector NTI (Invitrogen) confirmed successful

cloning. Once a successful pGEX-6P1-PS1 Loop construct had been created this was used as a template to create the following pGEX-6P1-PS1 Loop point mutants: pGEX-6P1-PS1 Loop F283A/P284A (pGEX-6P1-PS1 Loop FP/AA), pGEX-6P1-PS1 Loop V309A/S310A (pGEX-6P1-PS1 Loop VS/SS) and pGEX-6P1-PS1 Loop F283A/P284A/V309A/S310A (pGEX-6P1-PS1 Loop FP/VS/AA).

2.9 Purification of GST-Tagged Recombinant Proteins

The pGex 6P1-PS1 Loop, pGex 6P1-PS1 Loop FP/AA, pGex 6P1-PS1 Loop VS/AA and pGex 6P1-PS1 Loop FP/VS/AA expression vectors were transformed into BL21 cells and plated on LB agar plates containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. From each plate a single colony was picked and grown in 5 ml of LB broth containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol overnight at 37°C. Three ml of each culture was inoculated into 500 ml of LB Broth containing 50 μg/ml ampicillin and grown for 4-5 hours at 37°C until an OD_{595} reading of 1.0-2.0 had been reached. The cells were induced using 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown overnight at 20°C. The cultures were subject to centrifugation at 4000 rpm for 20 mins at 4°C. The resulting pellets were then resuspended in a lysis buffer [50 mM Tris (pH 8.5), 150 mM NaCl, 1 mM DTT with Complete protease inhibitors] (1 tablet per 50 ml of buffer). The cells were lysed through sonication in 10x1 minute cycles on ice using the Soniprep 150 (MSE). The GST-tagged PS1 Loop recombinant proteins were purified using a 2 hour incubation in a column containing glutathione agarose beads at 4°C. The column was washed with 2 L of washing buffer [50 mM Tris (pH 8.5), 500 mM NaCl, 1 mM DTT] and eluted from the column using elution buffer [50 mM Tris (pH 8.5), 150 mM NaCl, 10 mM Glutathione]. After the last of the wash buffer had passed through the column 25 ml off elution buffer was added to the beads. The

elution buffer was allowed to pass through the bead bed and was collected into 5 elutions of 5 ml volume. These elutions were further aliquoted into 500 µl volumes, then frozen on dry ice and stored at -80°C.

2.10 Cell Culture

Human Embryonic Kidney 293T (HEK293T) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM-21) containing 10% foetal bovine serum, 1% L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin. Murine embryonic fibroblasts (MEFs) were grown in Dulbecco's Modified Eagle's Medium (DMEM-21) containing 10% foetal bovine serum, 1% L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, non-essential amino acids, and sodium pyruvate. Cells were maintained in a humidified 37°C incubator with 5% CO_2. MEFs, PS1 deficient MEFs and presenilin double knock out MEFs were a gift from Prof. Bart De Strooper (KU Leuven, Belgium).

2.11 Calcium Phosphate Transfection of HEK293T Cells

Transfections were carried out on HEK293T cell cultures using the calcium phosphate precipitation method. For a 10 cm dish HEK293T cells were grown to 50% confluency before transfection. For each transfected 10 cm plate, 4 µg of DNA of each plasmid was used, to a maximum of 12 µg DNA. The transfection mixture consisted of the appropriate volume of DNA, the final volume was adjusted to 350 µl with autoclaved deionised water before 50 µl of filter sterilised 2 M $CaCl_2$ was added. This mixture was added dropwise to 400 µl of 2x Hank's Buffered Saline Solution (274 mM NaCl, 1.4 mM Na_2HPO_4, 42 mM HEPES pH 7.2). Then the DNA mixture was added dropwise over the entire surface of the 10 cm plate and put back into the incubator. After 12-16 hours the media was aspirated off and replaced with

fresh media. Cells were typically harvested 24-36 hours after transfection. For experiments with 6-well plates HEK293T cells were grown to 50% confluency before transfection. The reaction mixture was scaled down to a total volume of 200 µl, with 1 µg of each plasmid used per transfection up to a maximum of 2 µg DNA per well.

2.12 Transfection of Murine Embryonic Fibroblasts

Transfections in MEFs were carried out with Turbofect™. Twenty-four hours prior to transfection the cells were plated at $2x10^5$ in 6-well plates. For the transfection, 1 µg of plasmid DNA was mixed with 200 µl of serum free DMEM and allowed to stand for 5 mins. Then 3 µl of Turbofect was added and allowed to stand at room temperature for 20 mins. This mixture was then added dropwise over the entire surface of the well. The media was aspirated after 8-12 hours and replaced with fresh media. The cells were harvested 36 hours after transfection.

2.13 Western Blotting

When harvesting cells firstly the media was aspirated off and then the cells were washed twice in ice cold Phosphate buffered Saline (PBS) and then detached from plates by scraping with ice cold PBS supplemented with 5 mM Ethylenediaminetetraacetic acid (PBS-EDTA). The samples were then centrifuged at 2000 rpm for 5 minutes at 4°C. The supernatant was aspirated off and the cell pellets were re-suspended in RIPA buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl] supplemented with 1 mM sodium orthovanadate and Complete protease inhibitor cocktail (1 tablet was sufficient for 50 ml of buffer). The cells were lysed on ice for

50 minutes, centrifuged at 13000 rpm for 20 minutes at 4°C and then the supernatant was transferred to a fresh tube. The protein concentration of the lysate was determined using the BCA method. Proteins samples were diluted 1/10, mixed with 200 µl of BCA reaction mix (196 µl of BCA buffer A and 4 µl of BCA buffer B) and after 30 minute incubation at 37°C the samples were then read at 562 nm. To demonstrate equal efficiency of transfection, equivalent masses of proteins (50-80 µg) were resolved on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels before being transferred to nitrocellulose membrane. The membranes were blocked for an hour in 5% Marvel Milk in Tris-buffered saline (TBS) with 0.1% Tween (TBS-T). The membranes were incubated in the indicated primary antibody either overnight at 4°C or 2 hours at room temperature. After incubation in primary antibody the blots underwent 3x5 min washes with TBS-T before the blots were incubated with either ECL or Licor Odyssey secondary antibodies for 1-2 hours at room temperature. After incubation with the secondary antibody the blots were subjected to 3x5 min washes in TBS-T and then the proteins were detected using either the ECL method, according to manufacturers protocol, or using the Licor Odyssey CLx Imager system.

Table 2.2 Antibodies used for Western Blot Analysis and Immunoprecipitation.

Antibody	Supplier	Concentration	Species
Actin	Sigma-Aldrich	1/3000	Mouse
Anti-cleaved Notch (Val 1744)	Cell Signalling	1/1000	Rabbit
anti-FLAG	Sigma-Aldrich	1/3000 WB; 1/250	Mouse

		IP	
Anti-HA	Covance	1/1000 WB; 1/250 IP	Mouse
APP	Sigma-Aldrich	1/4000	Rabbit
IL-1R1 C20	Santa Cruz	1/250	Rabbit
IRDye 800CW anti-mouse	LiCor	1/10000	Goat
IRDye 800CW anti-rabbit	LiCor	1/10000	Goat
P4D1 anti-ubiquitin	Santa Cruz	1/1000	Mouse
P75NTR	Promega	1/1000	Rabbit
Polyclonal Goat anti-mouse HRP	Dako cytomation	1/5000	Rabbit
Polyclonal Goat anti-rabbit HRP	Dako cytomation	1/5000	Goat
PS1 CTF	Chemicon	1/1000	Mouse
PS1 NTF	Chemicon	1/300 IP	Rat
PS1 NTF 614.1	Scios	1/1000 WB; 1/250 IP	Mouse
PS2	Cell Signalling	1/1000 WB; 1/250 IP	Rabbit
RAB11	BD Bioscience	1/3000	Mouse
RIP1	BD Bioscience	1/1000	Mouse
TRAF2	Santa Cruz	1/250	Rabbit

2.14 Ubiquitination Assay

HEK293T cells were transiently transfected using the calcium phosphate method with the appropriate constructs. Thirty six hours post transfection the cells were washed with ice cold PBS and detached from plates by scraping in 1 ml PBS-EDTA (0.5 mM). Cells were pelleted by spinning at 2000 rpm for 5 min at 4°C. The cells were lysed in 250 µl of 1% SDS supplemented with protease inhibitors (Complete Roche Life Sciences) and then boiled for 5 min. Equal volume covalent buffer (50 mM Tris (pH 8), 150 mM NaCl, 1% Triton, 0.5% sodium deoxycholate and protease inhibitors) was added, the lysates were mixed and then spun at 13000 rpm for 30 min at 4°C. The pellets were removed and the lysates were normalised using the BCA method. Samples were immunoprecipitated by incubating the lysates with the appropriate antibody overnight at 4°C before protein-G sepharose beads (Roche Life Sciences) were added and the samples were incubated for a further 3 hours at 4°C. The beads were washed 3 times in covalent buffer before boiling in 2x SDS sample buffer. Precipitated proteins were resolved by SDS-PAGE and detected using the Licor Odyssey Infrared Imaging System.

2.15 Co-Immunoprecipitation Assay

HEK293T cells were transiently transfected 36 hours before harvesting. The media was aspirated from the cells, the plate was washed twice with ice cold PBS and then detached from the plate surface in 1 ml PBS-EDTA. The cells were pelleted by centrifugation at 2000 rpm for 5 mins at 4°C. The cell pellet was resuspended in lysis buffer [50 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM EDTA supplemented with Complete protease inhibitors (1 tablet per 50 ml of buffer) and 1 mM sodium orthovanadate]. Cells were lysed for 45 mins on ice, then centrifuged for 20 mins at 4°C. After centrifugation protein concentration was determined using the BCA

assay. After protein overexpression had been confirmed by western blotting, an equal mass of total protein from each sample were brought up to 1 ml volume with lysis buffer and then pre-cleared with 20 μg of protein-G sepharose beads for 1 hour at 4°C on the rotator. The beads were pelleted by centrifugation at 2000 rpm for 5 mins; the supernatants were transferred to a fresh tube and then subjected to immunoprecipitation (IP) with 3-4 μg of the specific antibody required. The IP samples were then incubated at 4°C overnight on the rotator. The following morning 30 μl of protein-G sepharose beads were added to each sample and incubated at 4°C on the rotator for 3 hours. The samples were then centrifuged for at 3000 rpm 2 mins, The supernatant was aspirated off and the beads were washed twice with 500 mM NaCl lysis buffer and once with 150 mM NaCl lysis buffer. After the final wash 40 μl of 2X Laemmli sample buffer [4% SDS, 20% glycerol, 120 mM Tris-HCl (pH 6.8), bromophenol blue 0.02% (w/v), 2% β-mercaptoethanol] was added to the beads and the samples were boiled for 5 mins. The beads were pelleted by centrifuging the samples at 13000 rpm for 0.5 mins and then the samples were resolved by SDS-PAGE on 12% gels. The co-immunoprecipitation of proteins was then detected by western blotting.

2.16 Ubiquitin Binding Assay

HEK293T cells were transiently transfected using the calcium phosphate method with the appropriate constructs. Thirty-six hours post transfection the cells were washed with ice cold PBS and detached from plates by scraping in 1 ml PBS-EDTA. Cells were pelleted by centrifugation at 2000 rpm for 5 min at 4°C. The cells were lysed in 250 μl of 1% SDS supplemented with protease inhibitors (1 tablet per 50 ml of buffer) and then boiled for 5 min. An equal volume of covalent buffer [50 mM Tris (pH 8), 150 mM NaCl, 1% Triton, 0.5% sodium deoxycholate, 1 mM sodium

orthovanadate and protease inhibitors] was added, the lysates were mixed and then centrifuged at 13000 rpm for 30 min at 4°C. The pellets were removed and the lysates were normalised using the BCA method. Samples were immunoprecipitated by incubating the lysates with the appropriate antibody overnight at 4°C before 30 µl of 50% protein-G sepharose beads were added and the samples were incubated for a further 3 hours at 4°C. The beads were washed 3 times in covalent buffer before boiling in 2X SDS sample buffer. At this stage the beads were washed once with binding buffer [20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Then 1 ml of binding buffer and 5 µg of recombinant (K-48 or K-63 linked) polyubiquitin were added to each sample and incubated on a rotator overnight at 4°C. The samples were centrifuged at 3000 rpm for 2 minutes and then washed 3 times with binding buffer. 30 µl of 2x sample buffer was added; the samples were boiled for 5 minutes and then centrifuged at 13000 rpm for 0.5 minutes. The samples were then resolved on a 12% SDS-PAGE gel and detected via western blotting.

2.17 Ubiquitin Pulldown Assay

HEK293T cells were transiently transfected with either HA-tagged $K48_{only}$ or $K63_{only}$ ubiquitin and then lysed in 1 ml of binding buffer [20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The GST-tagged PS1 Loop proteins were bound to 30 µl of glutathione agarose beads in binding buffer on a rotator for 3 hours. The glutathione agarose bound GST-PS1 Loop proteins were washed three times in binding buffer and then incubated with 800 µg of cell lysates from the HEK293T cells expressing either HA-tagged $K48_{only}$ or $K63_{only}$ ubiquitin. The samples were washed three times in binding buffer, 30 µl of 2x sample buffer was

added to the beads and then boiled for 5 minutes. The samples were then resolved on 12% SDS-PAGE gels. The gels were transferred to nitrocellulose paper and then ubiquitin binding was analysed by western blotting.

2.18 GST-Pulldown of Endogenous Mammalian Proteins for Mass Spec Analysis

10 x 15 cm dishes of HEK293T cells were grown to 100% confluency. The media was aspirated off and the cells were washed with 3 ml of ice cold PBS. The cells were harvested from the plates by scraping in PBS-EDTA and then pelleted at 4°C before aspirating off the PBS-EDTA. The cells were resuspended in 1.5 ml of binding buffer (with or without 8 mM phenanthroline) per plate used. The cells were then sonicated for 5x10 second cycles on ice before centrifuging at 15000 rpm for 30 min at 4°C and then protein concentration was determined using the BCA method. For the pull-down, 30 µl of 50% glutathione agarose slurry per 1 mg of protein was incubated with 30 mg with either GST-PS1 Loop or GST-PS1 FP/VS/AA Loop for 1 hour at room temperature. The GST recombinant protein buffer was then allowed to flow through. The HEK293T lysate was then added to the column and then incubated at 4°C overnight on the shaker. After the unbound lysate was allowed to flow through the beads were washed in 10 x bed volume of ice cold binding buffer. This step was repeated four times. Bound protein was eluted from the column by elution with 300 µl of an elution buffer (50mM Tris pH 8.5, 150 mM NaCl, 50 mM Glutathione) or by boiling the beads with 250 µL of 2X Laemmli Sample Buffer followed by centrifugation for 5 mins at 2000 rpm at 4°C. Protein binding was determined via separation on 12% SDS-PAGE followed by Coomassie staining. Gels were soaked in fixing solution (50% methanol, 10% glacial acetic acid, 40% H_2O) for 30 mins before 5 mins staining in Coomassie stain (50% methanol, 10%

glacial acetic acid, 40% H_2O, 0.25% Coomassie blue R-250). Gels were then washed in destaining solution (40% methanol, 10% glacial acetic acid, 50% H_2O) for 2-24 hours prior to scanning.

2.19 Mass Spectrometry

The mass spectrometry (MS) analysis was carried out by Fingerprints Proteomics (University of Dundee, UK) [Mutsaers *et al* 2013]. A one dimensional Liquid chromatography-MS-MS reverse phase chromatography approach was used to analyse the proteins on a 4000 QTRAP machine. 28 µl of each sample was mixed with 4 µl of N-Ethylmaleimide and 8 µl of 5X Laemmli sample buffer and loaded on to premade SDS-PAGE gels in a laminar flow. The protein bands were excised from the gel, gel purified and then digested with trypsin before analysis by MS [Walsh *et al* 2002]. The MS results were analysed and scored using the Mascot search engine (http://www.matrixscience.com/).

3 Characterisation of the Presenilin CUE Domains

Some of these data was previously published in FEBS Letters [Stephen P. Duggan, Run Yan, and Justin V. McCarthy (2015)] “A ubiquitin-binding CUE domain in presenilin-1 enables interaction with K63-linked polyubiquitin chains” FEBS Letters Apr 13;589(9):1001-8. doi: 10.1016/j.febslet.2015.03.008.

Introduction

PS1 and PS2 both contain a large hydrophilic intracellular loop domain between transmembrane domains 6 and 7. The intracellular loop domains are the regions that show the greatest difference between the sequences of the two presenilin proteins. While the overall ammino acid sequences of the human presenilins are 65% identical, the intracellular loop domain of PS2 is 19 residues shorter with the remainder of the loop being only 59% identical in sequence to that of PS1.The intracellular loop domains contain the sites of endoproteolysis where the catalytically inactive presenilin holoproteins are cleaved into their mature NTF and CTF fragments, which facilitate assembly and formation of the γ-secretase protease complexes. This intracellular loop domain has also been shown to be the site of protein-protein interactions between the presenilins and other proteins such as Rab11 [Dunmanchin et al 1999], syntaxin 5 [Smith et al 2000], β-catenin and δ-catenin [Zhou et al 1997] [reviewed in McCarthy et al 2009]. The interaction between PS1 and β-catenin and δ-catenin regulates Wnt signalling, demonstrating that the intracellular loop domain of PS1 is also involved in the γ-secretase independent roles of PS1. The PS1 intracellular loop domain is the site of a number of phosphorylation events driven by kinases such as PKA, PKC, GSK3β, JNK and CDK5, which regulate proteolysis of the presenilins and interactions with other intracellular proteins [Fluhrer *et al* 2004, Walter *et al* 1999, Kirschenbaum *et al* 2001, Kuo *et al* 2008, Lau et al 2002]. Furthermore, studies in our lab have shown that the intracellular loop domain of PS1 contains a TRAF6 interaction motif (PEERGV) at residues 374-379 [Elzinga et al 2009]. This interaction has been shown to influence the γ-secretase mediated cleavage of $P75^{NTR}$ [Powell et al 2009]. TRAF6 has also been shown to facilitate the K63-linked polyubiquitination of PS1 [Yan et al 2013;

Gudey *et al* 2014], though this ubiquitination has not been attributed to a particular lysine residue and TRAF6 seems to be able to ubiquitinate PS1 at multiple residues [Yan unpublished data]. This suggests that the large intracellular loops of the presenilins are involved in both γ-secretase dependent and independent roles of the presenilins and that they are the site of both post-translational modifications and protein-protein interactions. As such, studying and characterising conserved and divergent regions within the hydrophilic loop domains of PS1 and PS2 present an opportunity to investigate established and novel presenilin functions.

Previous members of our lab have used a bioinformatics approach to investigate the function of the intracellular domains of the presenilin proteins [Powell *et al* 2009]. Using this approach they showed that the presenilin proteins exhibited some sequence homology with proteins containing ubiquitin binding CUE domains. CUE domains from different proteins have been reported to facilitate binding to different types of ubiquitin, mono-, di- and polyubiquitin. For example, CUE domains have been shown to interact with both monoubiquitin, in the case of Vps9 [Davies et al 2003] and polyubiquitin chains in the case of Cue1p [Bagola et al 2013]. On the other hand the CUE domain of gp78 has been shown to have no preference in binding to monoubiquitin, K48- or K63 -inked polyubiquitin chains [Liu et al 2012]. The ubiquitination state of some proteins, such as Vps9 and gp78, is controlled by their CUE domains [Davies et al 2003, Chen et al 2005]. Similarly CUE domains can also regulate the ubiquitination of other proteins, such as the CUE domain of Tollip which has been shown to influence β-catenin ubiquitination and Wnt signalling [Torun et al 2015]. The CUE domain of Cue1p has been shown to regulate the formation of K48-linked polyubiquitin chains. In Tollip binding of ubiquitin to its CUE domain causes Tollip to have a lower affinity for phosphoinositide and as

such the Tollip CUE domain acts a switch that regulates Tollip's interaction with plasma membranes [Mitra et la 2013]. Collectively, the findings from these research studies proposes that the presence of CUE domains does not confer a single function upon proteins. Additionally different CUE domains are involved in diverse signalling pathways such as Wnt signaling [Torun et al 2015], NFκB signaling [Kishida et al 2005] and in ER associated degradation (ERAD) [Bagola et al 2013].

In this chapter the presenilins will be shown to contain a functional ubiquitin binding CUE domain. The presenilin CUE domain shows a preference in binding to K63-linked over K48-linked polyubiquitin chains. The VS motif, but not the FP motif, of the PS1 CUE domain are required for PS1 CUE domain function. In addition, this chapter will show that the PS1 CUE domain is dispensable for a number of γ-secretase related PS1 roles. Mutation of the conserved CUE domain motifs had no effect on PS1 endoproteolysis, though deletion of the CUE domain eliminated this function. The PS1 CUE domain was shown to be dispensable for the γ-secretase mediated cleavage of APP, Notch and IL-1R1.

3.1 Identification of a putative CUE domain in presenilin proteins

Previous members of our lab discovered sequence homology between a region of the intracellular loop of the presenilin proteins and proteins which contain an ubiquitin binding CUE domain (James Powell) **(Figure 3.1A)**. The CUE domain is approximately 40 amino acids in length, has a three-helix tertiary structure and contains two well-conserved motifs [Hurley *et al* 2006]. These motifs are the strongly conserved phenylalanine-proline (FP) motif and the less well conserved di-leucine motif. In human presenilins the putative CUE domain is found at amino acids 271-314 in PS1 **(Figure 3.1B)** and between amino acids 277-320 in PS2 **(Figure 3.1C)**. Both presenilin proteins contain the FP motif at residues F283/P284 for human PS1 and F289/P290 for human PS2; instead of the di-leucine motif human PS1 has the residues V309/S310 and human PS2 has the residues L315/Q316 **(Figure 3.1A)**. To study the tertiary structure of the putative presenilin CUE domains these sequences were analysed using the Jnet Structure predicting software [www.compbio.dundee.ac.uk]. This software predicted that these sequences would form the typical 3-helix structure that is attributed to other CUE domains [Hurley *et al* 2006]. To further investigate the putative CUE domains of both PS1 **(Figure 3.1A)** and PS2 **(Figure 3.1C)** were aligned with the amino acid sequences of presenilin proteins across a number of species. These alignments show that the putative CUE domains of both presenilin proteins are well conserved across evolutionary time. The FP-motif is strongly conserved across all species examined for both PS1 and PS2 while the VS- and LQ-motifs are less strongly conserved for both proteins. Residues with the presenilin CUE domains associated with FAD are also shown **(Figure 3.1D)**.

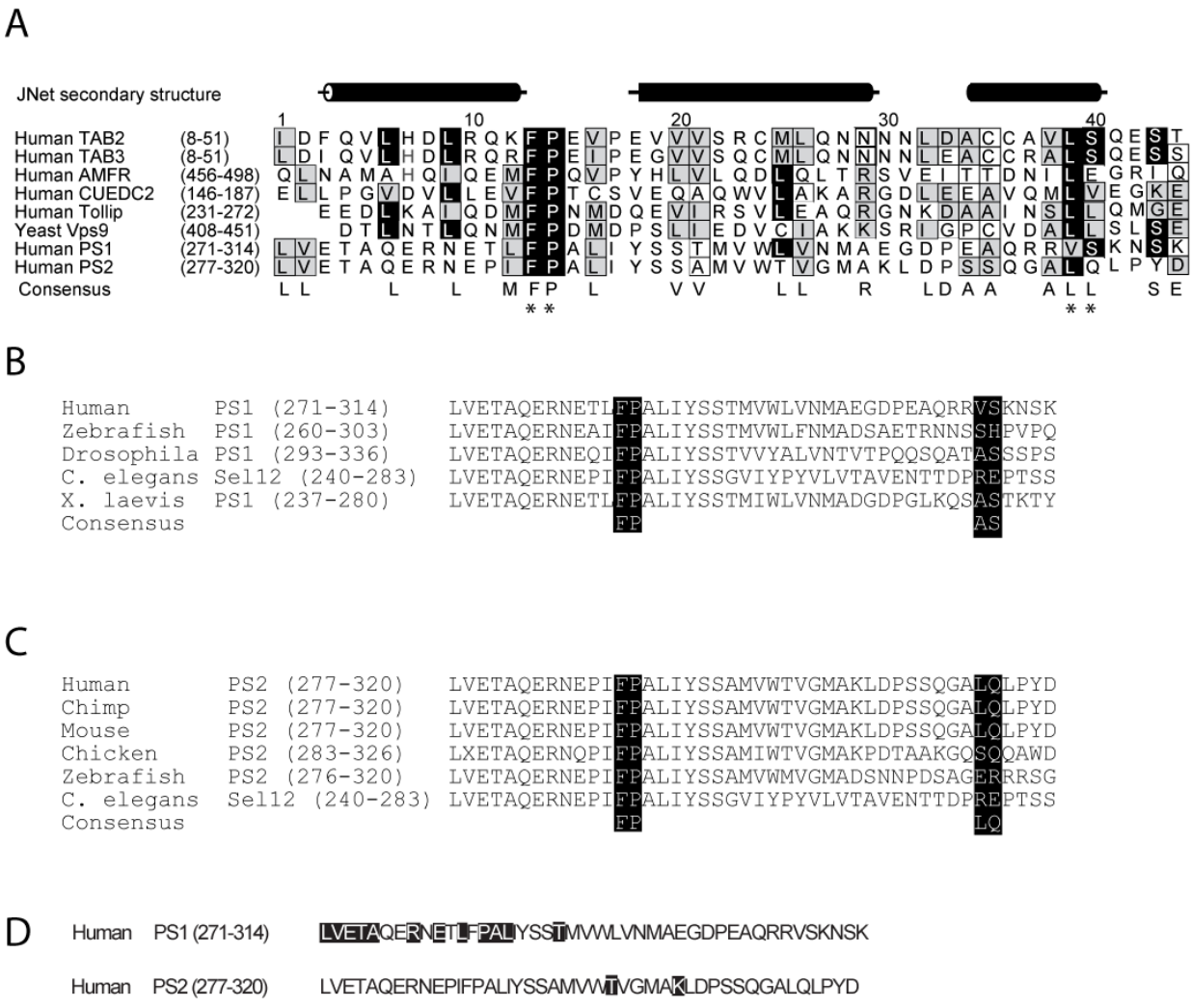


Figure 3.1 Alignment of the putative CUE domains of Presenilin proteins. (A) Shows an alignment of the putative CUE domains of the presenilin proteins with the sequence of known CUE domains. Residues highlighted in black are identical, residues highlighted in grey are similar and residues in boxes are weakly similar. Above the sequence alignment shows the position of the 3 helices predicted by the Jnet secondary structure prediction software. Below the alignment is the consensus sequence of the CUE domains present with the important FP and di-leucine motifs highlighted with stars. (B) and (C) show that the putative CUE domains of PS1 and PS2, respectively, are conserved across different species with the important CUE domain motifs highlighted in black. All alignments were done using AlignX software. (D) Schematic showing known familial Alzheimer's disease mutations within the CUE domains of the presenilins. Mutations are highlighted in black.

3.2 Validation of an Ubiquitin Binding Assay Using P62/Sequestosome 1

To investigate the ability of PS1 to bind to ubiquitin through its potential CUE domain an ubiquitin binding assay was first designed and optimised. It has previouly been shown that P62/sequestosome-1 contains a functional UBA domain that binds to K63-linked polyubiquitin chains [Seibenhener *et al* 2004], and a single point mutation within the UBA domain of P62, P62 F406V, abolishes ubiquitin binding to the P62 UBA domain. Using this information, first HEK293T cells were transiently transfected with pcDNA3.1 empty vector (EV), pcDNA3.1-HA-P62 or pcDNA3.1-HA-P62 F406V mutant. 236 hours post-transfection cell lysates were immunoprecipitated with an anti-HA antibody. Immunoprecipitates were subsequently incubated with equal concentrations (5 μg) of recombinant His-tagged lysine 63 (K63)-linked polyubiquitin chains. Ubiquitin binding was subsequently determined by SDS-PAGE and immunoblotting with an anti-polyubiquitin (P4D1) antibody. Consistent with previous studies, after immunoprecipitation wild type P62 but not P62 F406V bound to K63-linked polyubiquitin **(Figure 3.2)**. Loading control for recombinant lysine 63 (K63)-linked polyubiquitin used in experiment is indicated in the right hand side panel. These results demonstrate that this ubiquitin binding assay is able to differentiate between functional and dysfunctional ubiquitin binding domains and so can be used to test whether PS1 has a functional ubiquitin binding CUE domain.

3.3 PS1 Contains an Ubiquitin Binding CUE Domain

Having shown the validity of the ubiquitin binding assay, next this assay was used to investigate the ability of the PS1 CUE domain to bind to ubiquitin. To do this a PS1 mutant, called pcDNA3.1-PS1 ΔCUE, in which the entire CUE domain (residues 271-314) was deleted was used. This mutation also removed the site of PS1

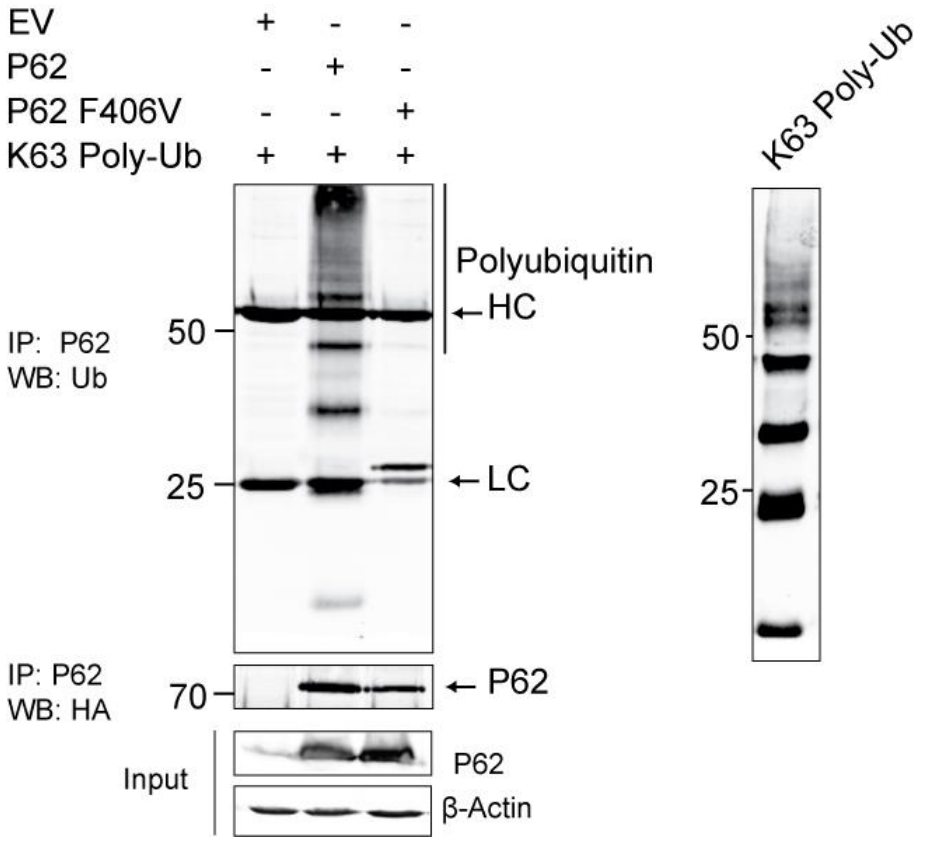


Figure 3.2 Validation of ubiquitin binding assay. HEK293T cells were transiently transfected with pcDNA3 (EV), pcDNA3.1-HA-P62 or the ubiquitin binding defective mutant pcDNA3.1-HA-P62 F406V and lysed under strict denaturing conditions. The lysates were subject to immunoprecipitation and then incubated with 5 μg of recombinant K63-linked polyubiquitin and bound ubiquitin was detected following SDS-PAGE and western blotting with P4D1 anti-ubiquitin antibody. The immunoprecipitation and expression of HA-P62 and HA-P62 F406V were confirmed by immunoblotting with anti-HA antibody. A β-actin blot is included to show loading control. The right panel shows a loading control with 1μg of recombinant K63-linked polyubiquitin used. IP: immunoprecipitation; WB: western blot; Ub: ubiquitin; HC: IgG heavy chain: IgG LC light chain; WT: wild type. Data are representative of experiments (n=3).

endoproteolysis at residue M292. HEK293T cells were transfected with pcDNA3.1, pcDNA3.1-PS1 or pcDNA3.1-PS1ΔCUE, and thirty six hours post-transfection cells were harvested under denaturing conditions and the cell lysates were immunoprecipitated with an anti-PS1 NTF antibody. The immunoprecipitates were then incubated overnight with equal quantities of recombinant His-taggedK63-linked or lysine 48 (K48)-linked polyubiquitin and after stringent washing ubiquitin-binding was determined by western blotting with an anti-polyubiquitin (P4D1) antibody. Equal amounts of PS1 full length protein were immunoprecipitated in this assay, whether it was PS1 FL or PS1 ΔCUE **(lower panel of Figure 3.3)**. The upper panel on left of Figure 3.3 demonstrates that PS1 has a preference for binding K63-linked over K48-linked polyubiquitin chains, as can be seen when you compare lane 2 to lane 4. In contrast, no binding to K63-linked polyubiquitin to PS1 was observed in the PS1 ΔCUE mutant. Loading control for recombinant lysine 63 (K63)-linked or lysine 48 (K48)-linked polyubiquitin used in experiment is indicated **(Figure 3.3, right panel)**.

3.4 Characterisation of the PS1 CUE Domain

Having shown that the human PS1 CUE domain contains a highly conserved FP and a less well-conserved VS motif that is shared with other CUE domains, it was decided to use a site-directed mutagenesis approach to generate mammalian expression constructs of the PS1 CUE missense mutants PS1 F283A/P284A and PS1 V309A/S310A to investigate PS1 CUE domain function. Previous studies demonstrated that mutation of the conserved methionine-phenylalanine-proline and di-leucine motifs reduce the ability of the CUE domain of Vps9 to interact with ubiquitin [Prag et al 2003]. The S310 residue of PS1 has previously been shown to

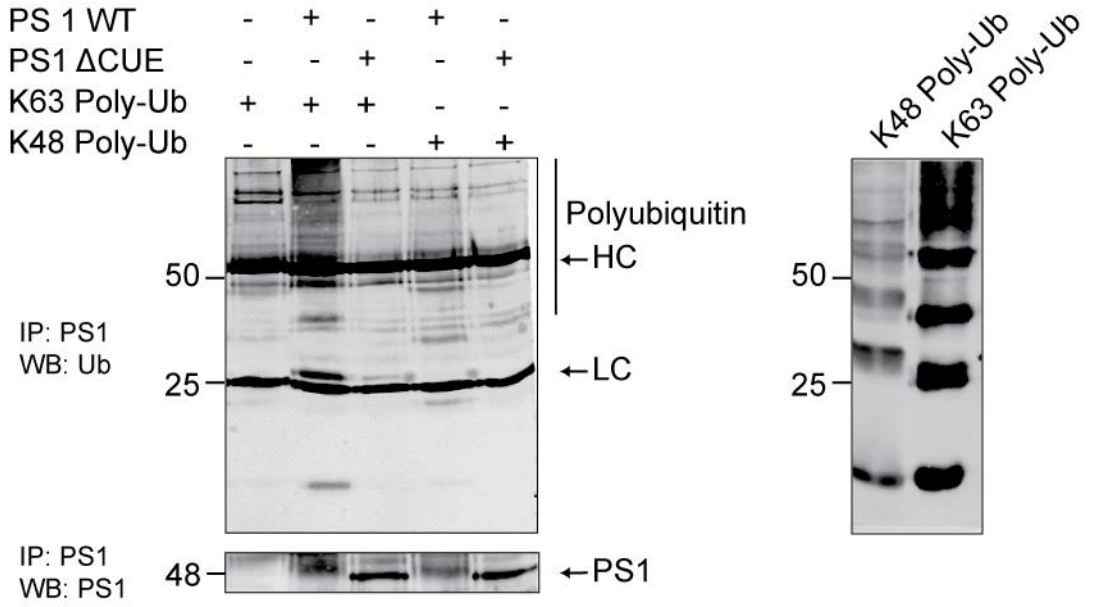


Figure 3.3 PS1 CUE domain selectively binds to K63-linked polyubiquitin chains. HEK293T cells were transiently transfected with pcDNA3, pcDNA3-PS1 WT or pcDNA3-PS1ΔCUE and lysed under strict denaturing conditions. The lysates were subject to immunoprecipitation and then incubated with 5 μg of either K63 or K48 recombinant polyubiquitin, as indicated above, and bound ubiquitin was detected following SDS-PAGE and western blotting with P4D1 anti-ubiquitin antibody. Equal immunoprecipitation was confirmed by immunoblotting with anti-PS1 CTF antibody. The right panel shows a loading control with 1ug of recombinant K63 and K48 polyubiquitin used. IP: immunoprecipitation; WB: western blot; Ub: ubiquitin; HC: IgG heavy chain: IgG LC light chain; WT: wild type. Data are representative of experiments (n=3).

be a site of PKA phosphorylation [Fluhrer 2004] and to investigate whether this could have any effect on PS1 CUE domain function a pseudo-phosphorylated PS1 S310E mutant was generated. HEK293T cells were transiently transfected with equal quantities of pcDNA3, pcDNA3-PS1, pcDNA3-PS1 ΔCUE, pcDNA3-PS1 F283A/P284A, pcDNA3-PS1 V309A/S310A or pcDNA3-PS1 S310E. The PS1 proteins were isolated using immunoprecipitation and were then incubated with 5μg of recombinant His-tagged K63-linked polyubiquitin **(Figure. 3.4A)**. Western blotting analysis revealed that wild-type PS1 and the PS1 F283A/P284A and PS1 S310E mutants bound to ubiquitin, while in contrast the PS1ΔCUE and PS1V309A/S310A mutants had reduced interaction with K63-linked polyubiquitin (compare lanes 4 and 6 with lane 3). These results suggest that the K63-linked polyubiquitin binding capability of the putative CUE of PS1 is not regulated by PKA activity but is mediated by the V309/S310 sequence. The F283/P284 motif does not appear to be as important in PS1 binding to K63-linked polyubiquitin as it shows a similar level of ubiquitin-binding to wild-type PS1. Figure 3.4B shows the overexpression of the pcDNA3-PS1, pcDNA3-PS1 ΔCUE, pcDNA3-PS1 F283A/P284A, pcDNA3-PS1 V309A/S310A and pcDNA3-PS1 S310E constructs in HEK293T cells that were used in the ubiquitin binding assay.

3.5 PS2 Contains a Functional Ubiquitin Binding CUE Domain

To investigate the functionality of the putative PS2 CUE domain a pcDNA3-PS2 ΔCUE mutant was created, where residues 277-320 of PS2 were deleted. HEK293T cells were transiently transfected with pcDNA3, pcDNA3-PS2 WT or pcDN3-PS2 ΔCUE **(Figure 3.5)**. Thirty-six hours after transfection the cells were harvested and lysed under strict denaturing conditions. Once transfection had been determined through western blotting (see Figure 3.5B) the lysates were subject to

immunoprecipitation with an anti-PS2 antibody. Once the immunoprecipitated proteins had been washed 3 times with covalent buffer and once with binding buffer the immunoprecipitated proteins were incubated overnight with either 5 μg of His-tagged K48- or 5 μg of His-tagged K63-linked polyubiquitin chains. After 3 washes with binding buffer the samples were boiled for 5 mins and then resolved on a 10% SDS-PAGE gel and the resulting blot was incubated with P4D1 anti-ubiquitin antibody. PS2 WT binds to K63 linked polyubiquitin but not K48 linked polyubiquitin chains **(compare lanes 2 and 4 in Figure 3.5)**. However, deletion of the PS2 CUE domain causes a reduction in binding to K63-linked polyubiquitin chains. The lower panel of Figure 3.5 shows that the PS2 ΔCUE proteins were not immunoprecipitated at a similar level to that of PS2 WT. However, the difference in ubiquitin binding to PS2 WT and PS2 Δ is greater than the difference in the immunoprecipitation of PS2 WT and PS2 ΔCUE. This ubiquitin binding assay also demonstrates that the PS2 CUE domain shows a preference for binding to K63-linked over K48-linked polyubiquitin chains. This shows that PS2 like PS1, contains a functional ubiquitin binding CUE domain that shows a preference for binding to K63-linked polyubiquitin over K48-linked polyubiquitin.

3.6 Characterisation of the PS2 CUE Domain

To further investigate the PS2 CUE domain a series of point mutants was created to replicate the mutants used in the PS1 *in vivo* ubiquitin binding assay. The mutants created were pcDNA3-PS2 F289A/P290A, and pcDNA-PS2 L315A/Q316A. HEK293T cells were transiently transfected with pcDNA3 (vector), pcDNA3-PS2 WT, pcDNA3-PS2 F289A/P290A, or pcDNA-PS2 L315A/Q316A for 36 hours. The

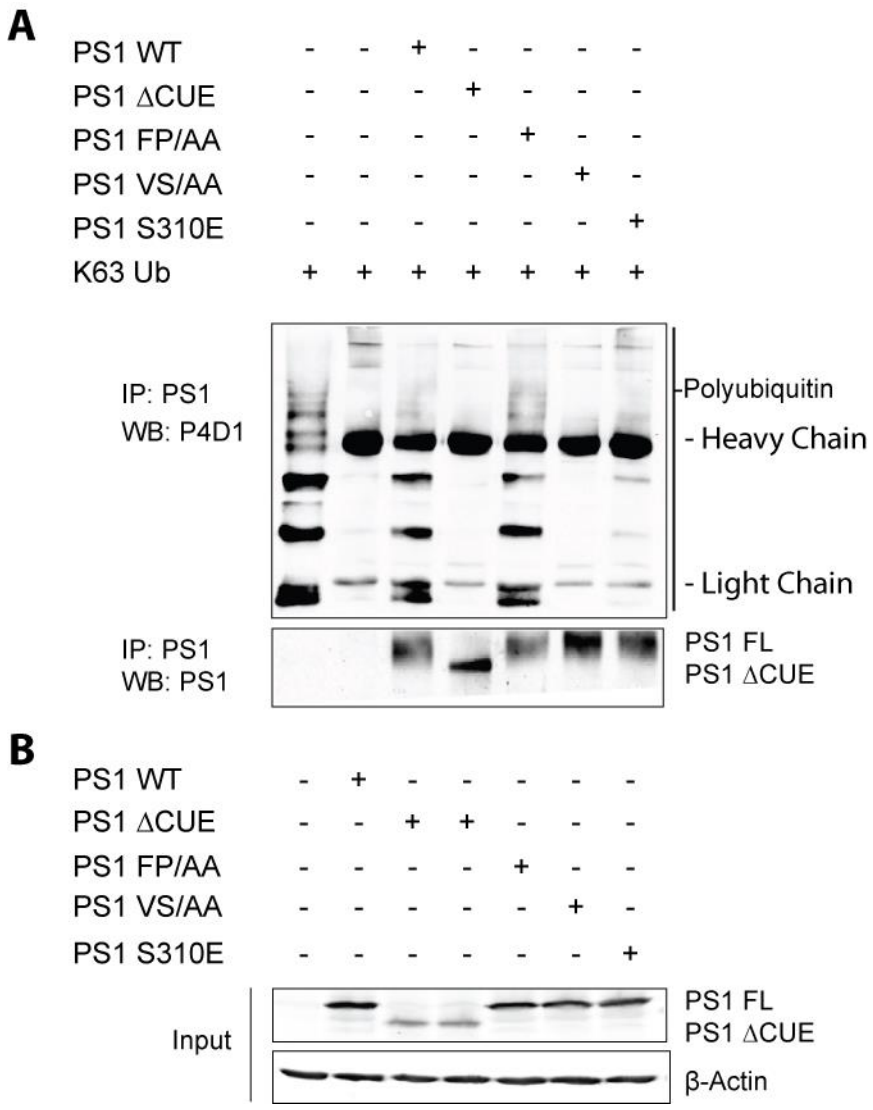


Figure 3.4 Characterisation of PS1 CUE domain mutants. HEK293T cells were transiently transfected with pcDNA3, pcDNA3-PS1 WT, pcDNA3-PS1ΔCUE, pcDNA3-PS1 F283A/P284A, pcDNA3-PS1 V309A/S310A or pcDNA3-PS1 S310E and lysed under strict denaturing conditions. (A) The lysates were subject to immunoprecipitation and then incubated with 5 μg of recombinant K63 polyubiquitin and bound ubiquitin was detected following SDS-PAGE and western blotting with P4D1 and anti-PS1 CTF. (B) The overexpression of pcDNA3-PS1 WT, pcDNA3-PS1ΔCUE, pcDNA3-PS1 F283A/P284A, pcDNA3-PS1 V309A/S310A or pcDNA3-PS1 S310E was confirmed by western blotting with anti-PS1 CTF antibody. A β-actin blot is included to show loading control. IP: immunoprecipitation; WB: western blot; Ub: ubiquitin; HC: IgG heavy chain: IgG LC light chain; WT: wild type; FL: full length. Data are representative of experiments (n=3).

cells were lysed under strict denaturing conditions and overexpression was examined by western blotting **(Figure. 3.6)**. Unlike the other plasmids used, the pcDNA-PS2 L315A/Q316A plasmid did not express in the HEK293T cells **(lane 6 of Figure 3.6)**. As a result of the poor expression of the pcDNA-PS2 L315A/Q316A plasmid, which is the PS2 equivalent of the ubiquitin binding defective PS1 V309A/S310A mutant, it was decided to focus more on the function of the PS1 CUE domain.

3.7 Generation of Recombinant GST-tagged PS1 Proteins

To further validate PS1 CUE domain function a GST-tagged recombinant protein containing the intracellular loop domain of PS1 was created. This protein contained residues 265-380 of PS1, contained within this sequence is the CUE domain of PS1 (residues 271-314). This recombinant protein was then used in an ubiquitin pulldown assay where the recombinant GST-tagged presenilin loop protein was incubated with lysates from HEK293T cells overexpressing ubiquitin constructs that can only form K48- or K63-linked polyubiquitin chains. Figure 3.7 shows a schematic representation of this approach, where the GST tag is on the N-terminal of each recombinant protein and the PS1 loop domain is at the C-terminal. In addition to the creation of a GST-PS1 Loop protein it was decided to use a mutagenesis approach to create a series of point mutants within the CUE domain to replicate the CUE domain mutants used in previous experiments. The F283/P284 and V309/S310 motifs of the PS1 CUE domain were either mutated singly or in tandem to create the following mutants: GST-PS1 FP/AA Loop (the recombinant version of the PS1 F283A/P284A mutant), GST-PS1 VS/AA (the recombinant version of the PS1 V309A/S310A mutant) and GST-PS1 FP/VS/AA (equivalent to a PS1 F283A/P284A/V309A/S310A mutant).

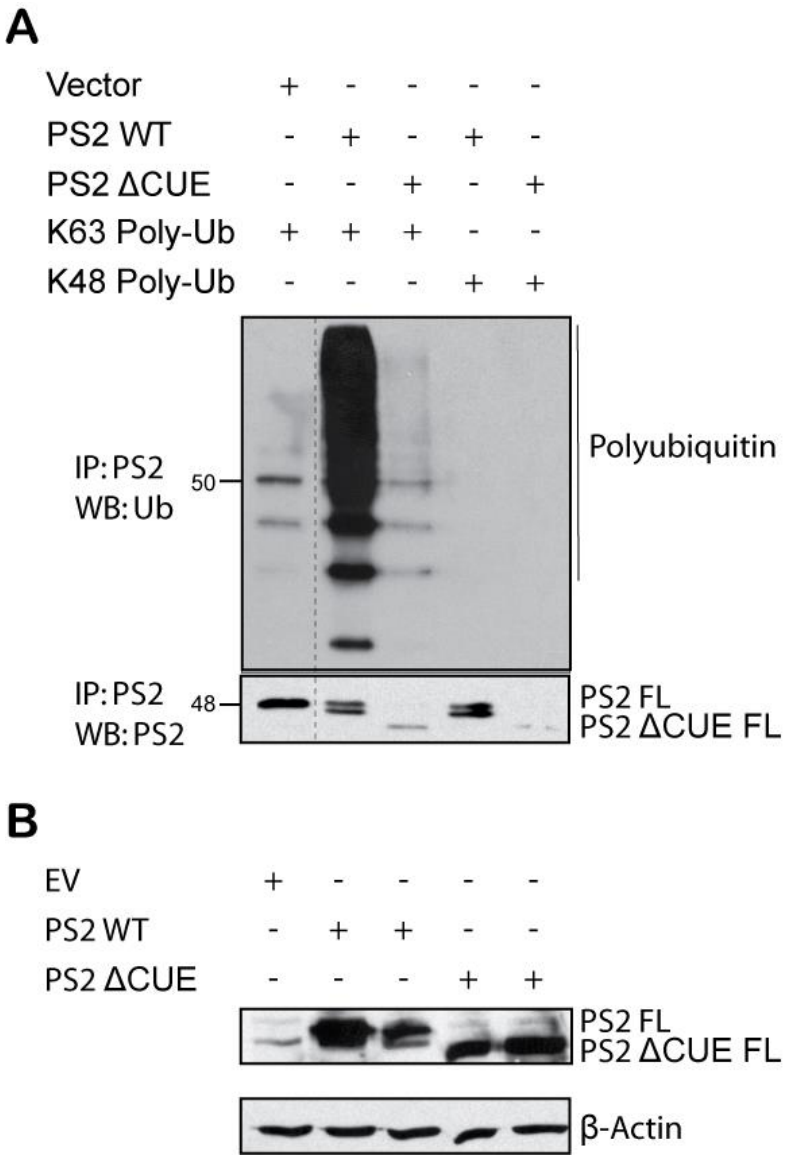


Figure 3.5 PS2 CUE domain selectively binds to K63 polyubiquitin chains. HEK293T cells were transiently transfected with pcDNA3, pcDNA3-PS2 WT or pcDNA3-PS2ΔCUE and lysed under strict denaturing conditions. (A) The lysates were subject to immunoprecipitation and then incubated with 5 μg of either K63 or K48 recombinant polyubiquitin, as indicated above, and bound ubiquitin was detected following SDS-PAGE and western blotting with P4D1 anti-ubiquitin antibody. Equal immunoprecipitation was confirmed by immunoblotting with anti-PS2 CTF antibody. (B) The overexpression of pcDNA3-PS2 WT and pcDNA3-PS2 ΔCUE was confirmed by western blotting with anti-PS2 CTF antibody. A β-actin blot is included to show loading control. IP: immunoprecipitation; WB: western blot; Ub: ubiquitin; HC: IgG heavy chain: IgG LC light chain; WT: wild type; FL: full length. Data are representative of experiments (n=2).

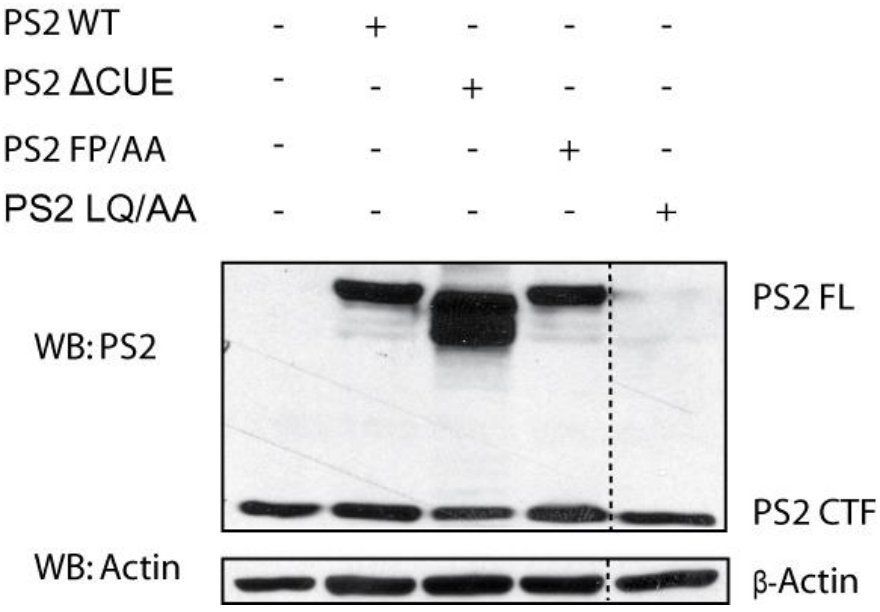


Figure 3.6 Characterisation of PS2 CUE domain. HEK293T cells were transiently transfected with pcDNA3, pcDNA3-PS2 WT, pcDNA3-PS2 ΔCUE, pcDNA3-PS2 F289A/P290A, or pcDNA3-PS2 L315A/Q316A and lysed under strict denaturing conditions. The overexpression of pcDNA3-PS2 WT, pcDNA3-PS2ΔCUE, pcDNA3-PS2 F289A/P290A, pcDNA3-PS2 F289S/P290S or pcDNA3-PS2 L315A/Q316A was confirmed by western blotting with anti-PS2 CTF antibody. A β-actin blot is included to show loading control.. WB: western blot; WT: wild type; FL: full length. Data are representative of experiments (n=1).

3.8 Purification of GST and recombinant GST-PS1 Loop Proteins

As outlined above (section 3.7) a series of GST-tagged human PS1 Loop constructs were generated. The pGEX-6P1 vector contains the gene for a bacterial GST protein and this protein was used as a negative control for any experiment that used these GST-tagged PS1 Loop recombinant proteins. pGEX-6P1 and pGEX-6P1-PS1 Loop plasmids were transformed into BL21 *E. coli* cells, a single colony was picked from either plate and grown in a 5 ml culture. This culture was expanded to a 500 ml culture before being induced with 0.5 mM IPTG and grown overnight. The cultures were centrifuged and the pellets lysed using sonication and the recombinant proteins were purified using a glutathione-agarose column. To demonstrate protein purification, samples taken during the purification process were separated on a 12% SDS-PAGE gel and then stained with Coomassie dye. The bacterial GST protein was expressed and was resolved at a size of 25 kDa on the SDS-PAGE gel **(Figure 3.8A)**. The five GST elutions can be seen in lanes 8-12 of the gel. The GST-PS1 Loop protein resolved at a size of 40 kDa **(Figure 3.8B)** and the six GST-PS1 Loop protein elutions can be seen in lanes 8-13. The ~15 kDa size difference between the two proteins represents the size of the inserted human PS1 loop domain.

3.9 Purification of GST tagged PS1 Loop CUE Domain Mutants

Once the pGEX-6P1-PS1 Loop plasmid had been created and the GST-PS1 Loop protein was successfully purified it was decided to make a series of recombinant PS1 CUE domain mutants. The pGEX-6P1-PS1 Loop plasmid was used as a template for site directed mutagenesis to create the following mutants: GST-PS1 Loop

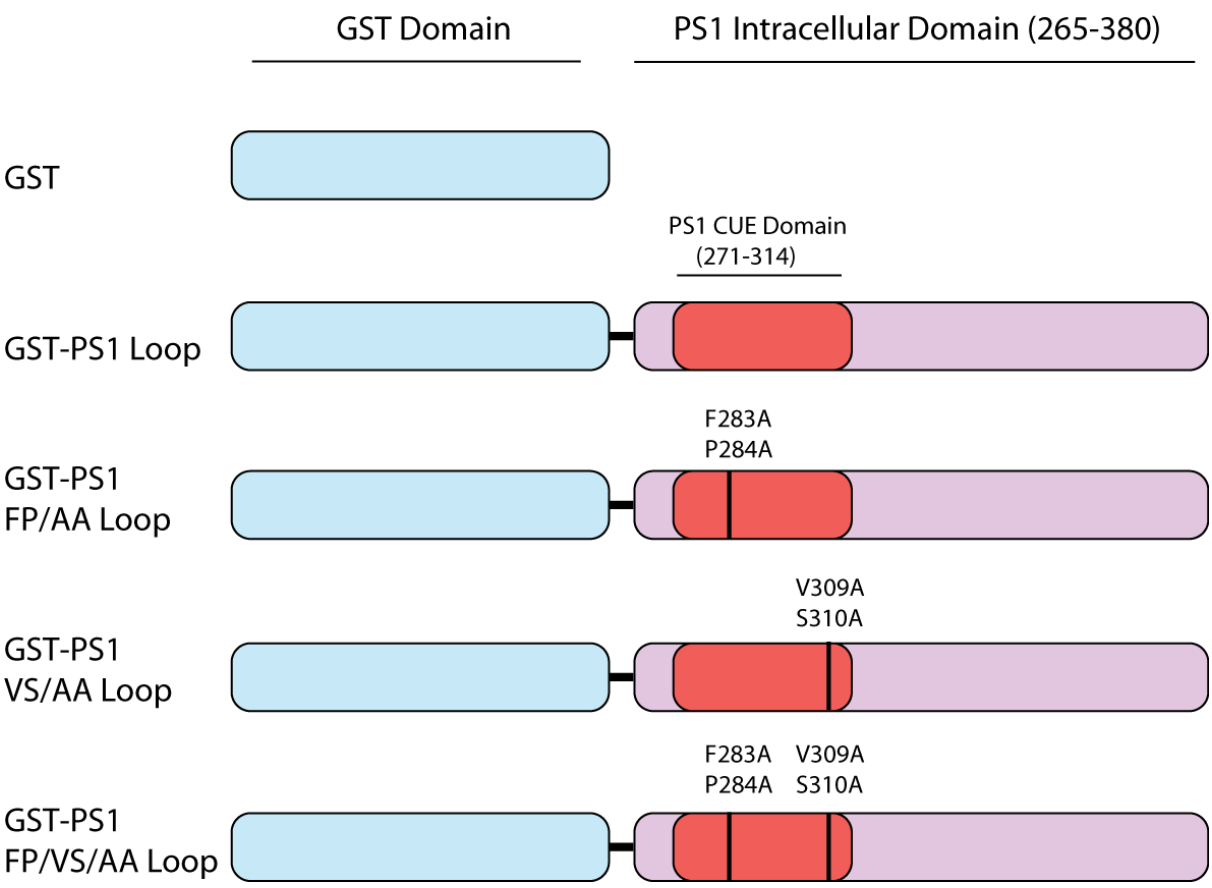


Figure 3.7 Schematic representation of recombinant GST- PS1 Loop proteins. The intracellular Loop domain (residues 265-380) of human PS1 was amplified by PCR and cloned into the pGEX-6P1 expression vector to give it a N-terminal GST tag (blue highlighted region). Within the intracellular loop domain (purple highlighted region) of PS1 is the 44 amino acid long CUE domain (red highlighted region). Using site-directed mutagenesis the two key motifs within the PS1 CUE domain (positions marked above) were mutated singly or in tandem to create the following mutant proteins: pGEX-6P1 GST-PS1 Loop FP/AA, pGEX-6P1 GST-PS1 Loop VS/AA and pGEX-6P1 GST-PS1 Loop FP/VS/AA.

F283A/P284A (GST-PS1 Loop FP/AA), GST-PS1 Loop V309A/S310A (GST-PS1 Loop VS/AA) and GST-PS1 Loop F283A/P284A/V309A/S310A (GST-PS1 Loop FP/VS/AA). These plasmids were transformed into BL21 *E. coli* cells, a single colony was picked from each plate and grown in 5 ml of LB broth. This culture was expanded into a 500 ml culture before being induced with 0.5 mM IPTG and the culture was grown overnight. The culture was centrifuged, lysed by sonication and the GST-tagged recombinant proteins were purified on a glutathione-agarose column. The recombinant proteins were eluted with an elution buffer containing 10 mM glutathione. Samples were taken during the purification process of each recombinant protein and resolved using a 12% SDS-PAGE gel. After separation the gel was stained with coomassie dye. The gel of the GST-PS1 Loop purification process from Figure 3.8B was repeated here to show equivalency of protein purification of the recombinant proteins. Figure 3.9B shows the purification process of the GST-PS1 Loop FP/AA protein, the induced recombinant protein can be seen at 40 kDa as before and the 4 elutions of the GST-PS1 Loop FP/AA protein can be seen in lanes 8-11. The recombinant proteins present in these elutions were later used in an ubiquitin pulldown assay. The purification of GST-PS1 Loop VS/AA protein can be seen in Figure 3.9C and the 5 elutions can be seen in lanes 8-12. The purification of the doubly mutated GST-PS1 Loop FP/VS/AA protein can be seen in Figure 3.9D and the elutions can be seen in lanes 8-12. Each elution of the recombinant GST-PS1 loop proteins were broadly similar to one another **(Figure 3.9)**. To demonstrate the equivalency of the elutions to one another 10 µl volumes of elution 2 of the GST, GST-PS1 Loop, GST-PS1 Loop FP/AA and GST- PS1 Loop VS/AA proteins were run on 12% SDS-PAGE gels.

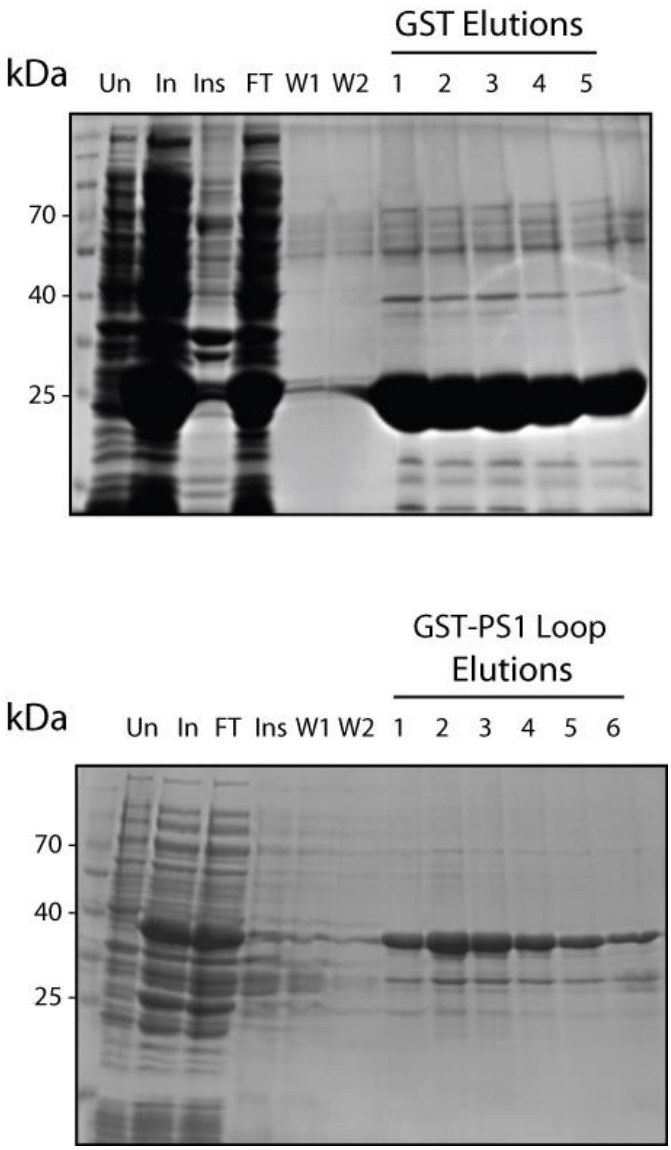


Figure 3.8 Purification of recombinant GST and GST-PS1 Loop proteins. pGEX-6P1 and pGEX-6P1-PS1 Loop were transformed into BL21 E.coli cells and the culture expanded to 500 ml before induction for 16 hrs with IPTG. The cultures were lysed through sonication and the recombinant proteins were purified using a glutathione-agarose column. Samples were taken during the different purification stages and protein purification was demonstrated following separation through SDS-PAGE. Purification of (A) recombinant GST and (B) recombinant GST-PS1 Loop were shown through staining with a Coomassie dye.. kDa: kilodaltons; Un: uninduced; In: induced; Ins: insoluble; FT: flow through; W: wash.

GST-PS1 Loop and GST-PS1 Loop FP/AA were purified at roughly similar levels, which was roughly double the amount of purified GST-PS1 Loop VS/AA **(Figure 3.9E)**. There was roughly 4 times as much GST protein purified as compared to GST-PS1 Loop. Figure 3.9F shows the comparison between elution 2 of GST-PS1 Loop and all elutions of GST-PS1 Loop FP/VS/AA. This demonstrates that elutions 1, 2 and 4 are roughly 1/3 the intensity while elution 3 is roughly 1/2 intensity of GST-PS1 Loop.

3.10 Recombinant GST-PS1 Loop and GST-PS1 Loop FP/AA but not GST-PS1 Loop VS/AA Mutant Binds to K63-Linked Polyubiquitin

Having created that series of recombinant GST proteins (GST, GST-PS1 Loop, GST-PS1 Loop FP/AA, GST-PS1 Loop VS/AA and GST-PS1 loop FP/VS/AA), they were next used to further demonstrate the functionality of the PS1 CUE domain. Instead of using the His-tagged recombinant proteins that were used in the *in vivo* ubiquitin binding assay **(Figure 3.3)**, it was decided to use ubiquitin proteins expressed in HEK293T cells. Firstly, GST, GST-PS1 Loop, GST-PS1 Loop FP/AA and GST-PS1 Loop VS/AA recombinant proteins were immobilized with 30 µl of glutathione agarose beads, following incubation for 2 hours at 4°C. Next the immobilized recombinant GST-PS1 proteins were incubated with lysates from HEK293T cells that were over expressing HA-tagged $K63_{only}$ ubiquitin (HA-Ub $K63_{only}$). This ubiquitin mutant has every lysine residue mutated to arginine except for the lysine 63 (K63) residue and so it can only form K63-linked polyubiquitin chains. GST protein was used as negative control for this experiment. The resulting samples were run on a 12% SDS-PAGE gel and the presence of bound HA-tagged ubiquitin was detected by immunoblotting for HA. Consistent with data presented in Figure 3.4A, the GST-PS1 Loop domain fusion protein and GST-PS1 Loop FP/AA

mutant bound to K63-linked polyubiquitin, while the GST-PS1 Loop VS/AA domain mutant lost the ability to bind K63-linked polyubiquitin **(Figure 3.10A)**. To verify PS1 selectivity for binding to K63-linked ubiquitin in our pull-down assay, recombinant GST-PS1 Loop domain protein and GST-PS1 Loop VS/AA mutant were immobilized on glutathione agarose beads and then incubated with cell lysate from HEK293T cells overexpressing HA-tagged $K63_{only}$ ubiquitin (HA-Ub $K63_{only}$) or HA-tagged $K48_{only}$ ubiquitin (HA-Ub $K48_{only}$) **(Figure 3.10B)**. The GST-PS1 Loop domain fusion protein selectively bound to K63-linked polyubiquitin and not K48-linked polyubiquitin, while the GST-PS1 VS/AA mutant was again shown to have lost its ability to bind K63-linked polyubiquitin **(Figure 3.10B)**.

3.11 PS1 CUE Mutation Has No Effect On PS1 Endoproteolysis

Having shown that PS1 contains a functional ubiquitin binding CUE domain next it was determined whether the CUE domain was required for presenilin endoproteolysis and γ-secretase activity. Given that the CUE domain (residues 271–314) of PS1 is located within the large hydrophilic loop domain and spans the sites of presenilin endoproteolysis (PS1 Met292/Val293) [Jacobsen *et al* 1999; Thinakaran *et al* 1996], we examined the possible involvement of the putative CUE domain in endoproteolysis of PS1 by deletion of the entire CUE domain from PS1 (PS1 ΔCUE) and mutagenesis of the two conserved motifs within the PS1 CUE domain (PS1F283A/P284A and PS1V309A/S310A). Presenilin double knockout murine embryonic fibroblasts (PSDKO MEFs) were transfected with pcDNA3, pcDNA3-PS1 WT, the catalytically inactive pcDNA3-PS1D257A/D385A (PS1ΔAsp) mutant, pcDNA3-PS1 ΔCUE, or CUE domain point mutants pcDNA3-PS1 F283A/P284A or pcDNA3-PS1 V309A/S310A. Cell lysates were separated by SDS-PAGE and PS1

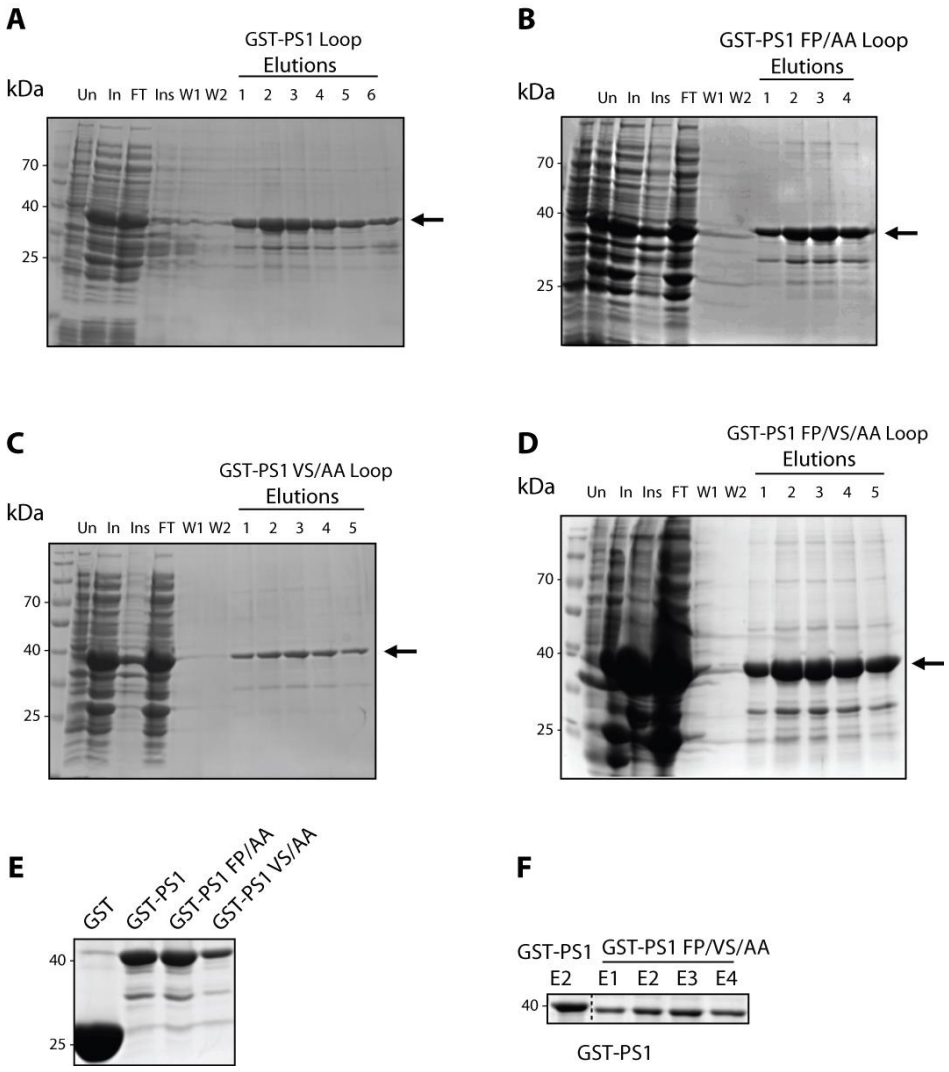


Figure 3.9 Purification of recombinant PS1 CUE domain mutant proteins. pGEX-6P1-PS1 Loop, pGEX-6P1-PS1 Loop FP/AA , pGEX-6P1-PS1 Loop VS/AA and pGEX-6P1-PS1 Loop FP/VS/AA were transformed into BL21 E.coli cells and the cultures expanded to 500 ml before induction for 16 hrs with IPTG. The cultures were lysed and the recombinant proteins were purified using a glutathione-agarose column. Samples were taken during the different purification stages and protein purification was demonstrated following separation through SDS-PAGE. Purification of (A) GST-PS1 Loop, (B) GST-PS1 FP/AA Loop, (C) GST-PS1 VS/AA Loop and (D) GST-PS1 FP/VS/AA Loop were shown through staining with a coomassie dye. The purified proteins are indicated by the arrows in the above gels. Elutions 2 of GST, GST-PS1 Loop, GST-PS1 Loop FP/AA and GST-PS1 Loop Vs/AA proteins are compared in (E). All GST-PS1 Loop FP/VS/AA are compared to GST-PS1 Loop elution 2 in (F). kDa: kilodaltons; Un: uninduced; In: induced; Ins: insoluble; FT: flow through; W: wash.

endoproteolysis was determined by immunoblotting with anti-PS1 NTF antibody **(Figure 3.11)**. As anticipated, expression of the catalytically inactive PS1D257A/D385A (PS1ΔAsp) mutant, or deletion of PS1 CUE domain prevented the endoproteolysis of PS1, and abolished the formation of PS1 NTF fragment **(Figure 3.11)**. However, expression of the PS1 CUE domain point mutants PS 1F283A/P284A and PS1 V309A/S310A had no effect on PS1 endoproteolysis and formation of PS1 NTF fragment.

3.12 PS1 CUE Domain Is Dispensable For γ-Secretase Cleavage of APP

The presenilin proteins are predominantly studied as a component of the γ-secretase protease complex involved in the regulated intramembrane proteolysis of APP, Notch and several other type I integral membrane proteins [McCarthy *et al* 2009]. To measure the effect of PS1 CUE domain deletion or mutation of the conserved PS1 CUE domain motifs on γ-secretase activity in cells PS1 CUE mutants were co-expressed with a truncated APP mutation (APP CT100-FLAG), which corresponds to the β-secretase generated APP C99 C-terminus fragment, which is a constitutive substrate for γ-secretase protease. First, HEK293T cells, were transiently transfected with APP CT100-FLAG plasmid and co-transfected with pcDNA3, PS1, PS1ΔCUE PS1 F283A/P284A or PS1 V309A/S310A and cell lysates were subjected to Western blot analysis with an anti-APP C-terminus-specific antibody **(Figure 3.12A)**. In cells expressing PS1, overexpressed APP C99-FLAG fragment is clearly detected **(Figure 3.12A, lane 3)**. In cells expressing PS1 and treated with the γ-secretase inhibitor, Compound E, accumulation of overexpressed APP C99-FLAG and endogenous APP C99 fragments are clearly detected **(Figure 3.12A, lane 4)**. In contrast, expression of

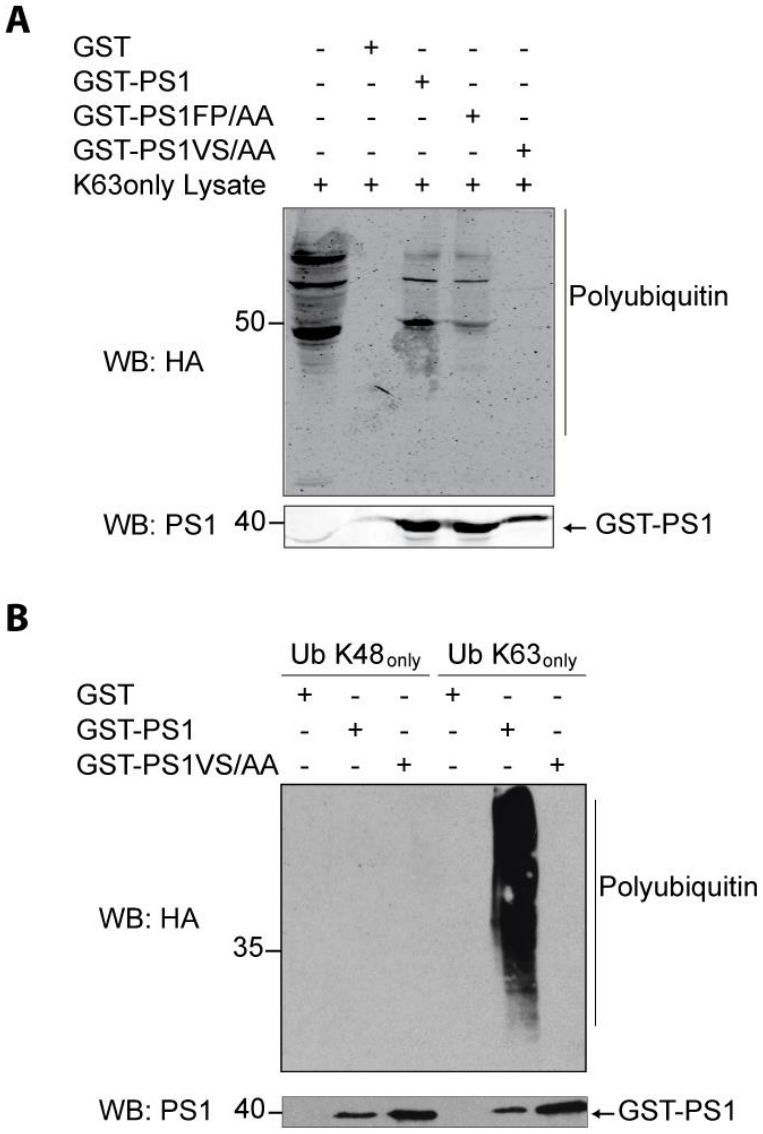


Figure 3.10 Recombinant GST-PS1 selectively binds to K63 polyubiquitin while the GST-PS1 VS/AA mutant does not. (A) Recombinant GST, GST-PS1 Loop, GST-PS1 FP/AA Loop and GST-PS1 VS/AA Loop were bound to glutathione-agarose beads and incubated with lysates from HEK293T cells overexpressing K63only HA-Ub. Bound protein was resolved by SDS-PAGE and ubiquitin binding was detected by western blotting with anti-HA antibody. Pulldown of GST-PS1 Loop, GST-PS1 FP/AA Loop and GST-PS1 VS/AA Loop was confirmed by western blotting with anti-PS1 CTF antibody. (B) GST, GST-PS1 Loop and GST-PS1 VS/AA Loop were bound to glutathione-agarose beads and incubated with lysates from HEK293T cells overexpressing K48only or K63only HA-Ub. Bound protein was separated via SDS-PAGE and bound protein was detected by western blotting with anti-HA antibody. Pulldown of GST-PS1 Loop and GST-PS1 VS/AA Loop was confirmed by western blotting with anti-PS1 CTF antibody. WB: western blot; Ub: ubiquitin. Data are representative of experiments (n=3).

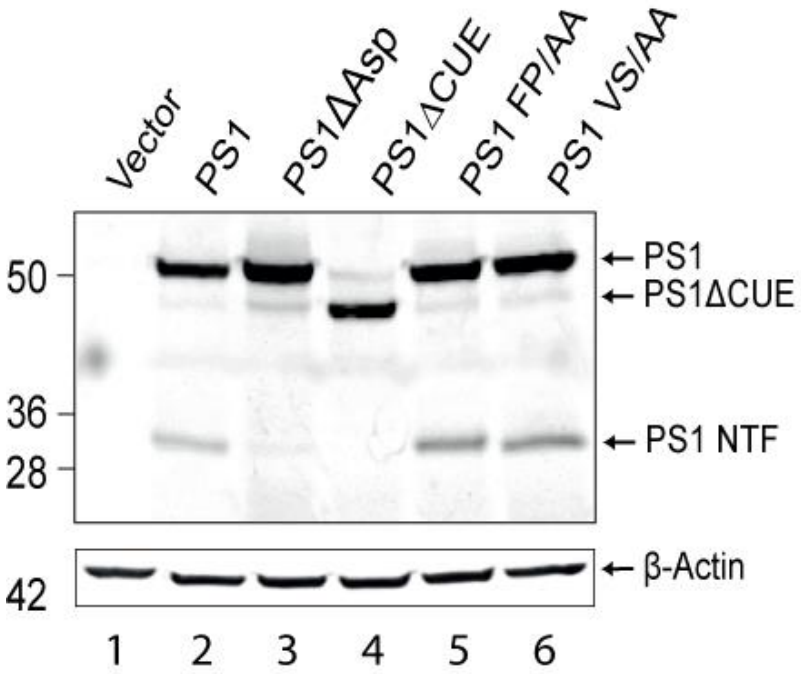


Figure 3.11 Mutation of PS1 CUE domain does not affect PS1 endoproteolysis. Presenilin double knock out MEFs were transiently transfected with pcDNA3, pcDNA3-PS1 WT, pcDNA3-PS1 ΔAsp, pcDNA3-PS1 ΔCUE, pcDNA3-PS1 F283A/P284A or pcDNA3-PS1 V309A/S310A expression vectors. Thirty six hours after transfection the cells were harvested, lysed with RIPA buffer and proteins were resolved by SDS-PAGE. PS1 endoproteolysis was detected by western blotting with 614.1 anti-PS1 NTF antibody. Equal loading was confirmed by western blotting with an anti-β-actin antibody. Data are representative of experiments (n=2).

PS1 CUE mutants did not alter levels of exogenous APP C99-FLAG nor endogenous APP C99 C-terminal fragment, which indicates that deletion of the CUE domain nor mutation of key residues does not induce a major change in γ-secretase activity **(Figure 3.12A)**. To further confirm the result with a more sensitive method, cell culture medium was collected after transfection and tested using ELISA to measure the concentration of the γ-secretase cleavage products Aβ40 and Aβ42 **(Figure 3.12B)**. Results showed that CUE domain deletion or mutagenesis does not induce significant change in the generation of soluble Aβ40 and Aβ42.

3.13 PS1 CUE domain is dispensable for γ-secretase cleavage of APP in MEFs

To further validate this observation **(Section 3.12)**, PS1-deficient MEFs were transiently transfected with pcDNA3, pcDNA3-PS1, pcDNA3-PS1ΔCUE, pcDNA-PS1 F283A/P284A or pcDNA3-PS1 V309A/S310A and cell lysates were analysed for endogenous APP C99, by Western blotting with an APP C-terminus-specific antibody **(Figure 3.13)**. Again, expression of the CUE mutants had no effect on levels of endogenous APP C99, whereas treatment with the γ-secretase inhibitor Compound E (lane 3) caused the accumulation of the APP C99 fragment, therefore demonstrating that loss of or mutagenesis of the PS1 CUE domain does not significantly alter γ-secretase cleavage of APP.

3.14 PS1 CUE domain is dispensable for γ-secretase cleavage of Notch

Notch is another major γ-secretase substrate that is essential for development [Geling *et al* 2002]. To further examine the functionality of the PS1 CUE domain in the cleavage of γ-secretase substrates, HEK293T cells were transiently transfected with NEXT, a Notch construct that is constitutively cleaved by γ-secretase, and co-

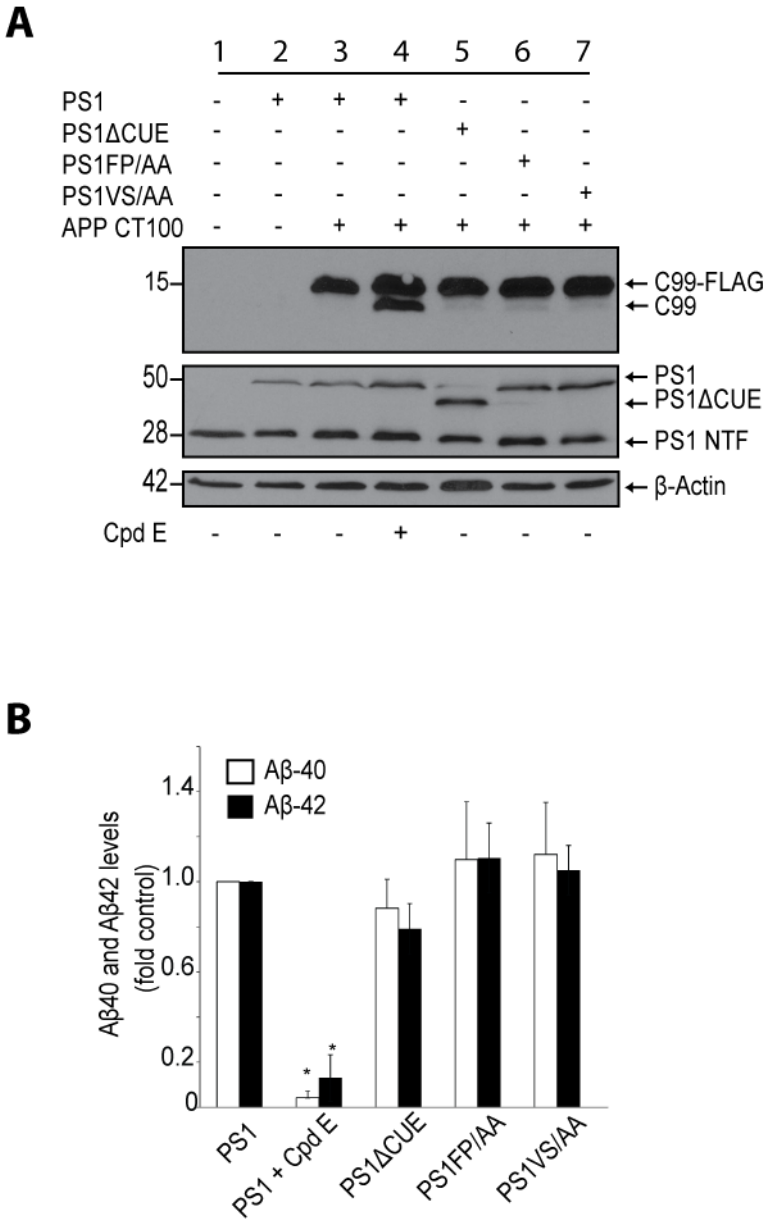


Figure 3.12 PS1 CUE domain is dispensable for γ-secretase cleavage of APP. (A) HEK293T cells were transiently co-transfected with FLAG-APP CT100 and pcDNA3-PS1 WT, pcDNA3-PS1 ΔCUE, pcDNA3-PS1 F283A/P284A or pcDNA3-PS1 V309A/S310A expression vectors. Twenty four hours after transfection cells were treated with the γ-secretase inhibitor compound E (Cpd E) (50 nM) as indicated. After 12 hours treatment with Cpd E the cells were harvested, lysed with RIPA buffer and the lysates were separated by SDS-PAGE. Cleavage of APP was detected by immunoblotting with an anti-APP CTF antibody; expression of the PS1 constructs was confirmed by immunoblotting with 614.1 anti-PS1 NTF antibody. A β-actin blot was included as a loading control. Data are representative of experiments (n=3). (B) Media from HEK293T cells co-transfected with FLAG-APP CT100 and pcDNA3-PS1 WT, pcDNA3-PS1 ΔCUE, pcDNA3-PS1 F283A/P284A or pcDNA3-PS1 V309A/S310A was collected and used in ELISAs for Aβ40 and Aβ42. Data shown are the mean (SEM) of 3 independent experiments and $p < 0.05$ is considered significant.

transfected with pcDNA3 (Vector), pcDNA3-PS1 WT, pcDNA3-PS1 ΔCUE, pcDNA3-PS1 F283A/P284A or pcDNA3-PS1 V309A/S310A. Twenty-four hours post-transfection cell were treated with the γ-secretase inhibitor Compound E. After 12 hours treatment with Compound E the cells were lysed and subjected to Western blot analysis with an anti-cleaved Notch 1 specific antibody **(Figure. 3.14)**. In cells expressing PS1, generation of NICD was evident **(Figure 3.14, lane 3)**, while in cells treated with the γ-secretase inhibitor, compound E **(Figure 3.14, lane 4)** formation of NICD was inhibited. In contrast, in cells expressing PS1 ΔCUE, PS1 F283A/P284A or PS1 V309A/S310A, NICD formation is still observed. However, co-expression of NEXT with the pcDNA-PS1 ΔCUE mutant does cause a reduction in the formation of cleaved notch suggesting that deletion of the PS1 CUE domain may have an effect on modulating γ-secretase cleavage of Notch. These data shows that mutagenesisof key motifs within the CUE domain of PS1 has no effect on the γ-secretase mediated cleavage of Notch.

3.15 PS1 CUE domain has no effect on γ-secretase cleavage of IL-1R1

There are over 100 known γ-secretase substrates known [McCarthy et al 2009] with APP and Notch being the most examined. As shown above deletion or mutation of the PS1 CUE domain has no effect on the cleavage of these two γ-secretase substrates. However, ubiquitination has been shown to regulate the γ-secretase mediated cleavage of some substrates [Gudey *et al* 2014, Twomey *et al* 2009]. Previous work in our lab has shown that IL-1R1 is a γ-secretase substrate and that this cleavage event is mediated by the E3 ligase TRAF6 [Elzinga *et al* 2009]. TRAF6 has also been shown to cause the K63-linked polyubiquitination of IL-1R1, which positively influences its cleavage by the γ-secretase complex [Twomey *et al* 2009]. As the PS1 CUE domain has been shown to selectively bind to K63-linked

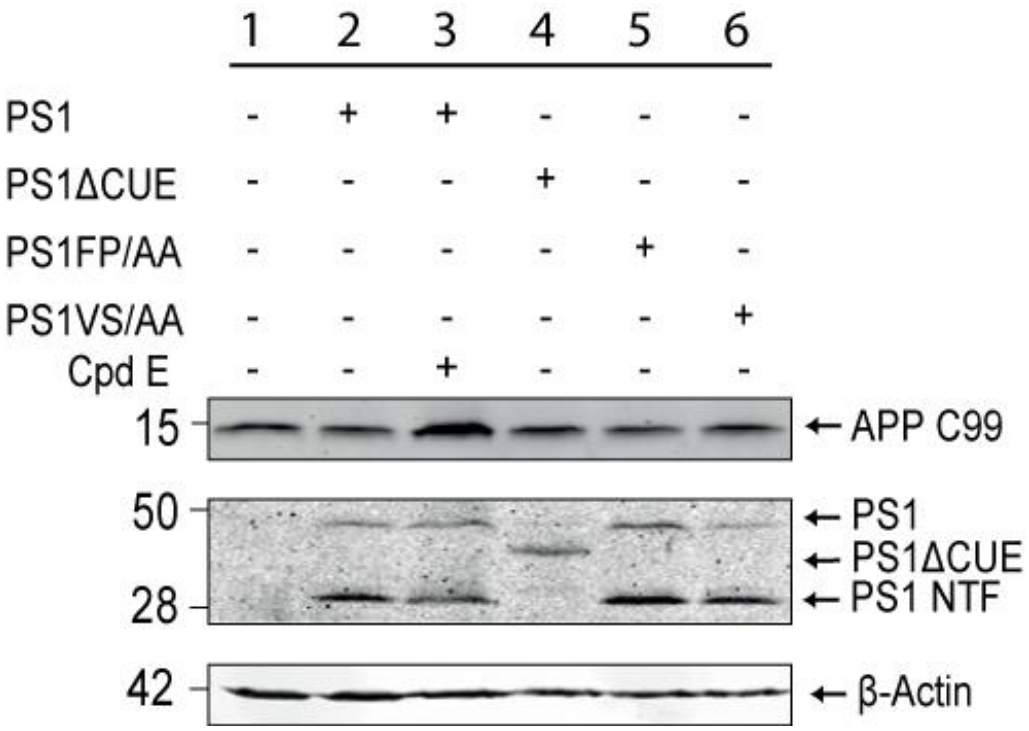


Figure 3.13 PS1 CUE domain function is dispensable for γ-secretase of APP cleavage in PS1KO MEFs. PS1KO MEF cells were transiently transfected with pcDNA3, pcDNA3-PS1 WT, pcDNA3-PS1 ΔCUE, pcDNA3-PS1 F283A/P284A or pcDNA3-PS1 V309A/S310A expression vectors. Twenty four hours after transfection cells were treated with the γ-secretase inhibitor compound E (Cpd E) (50 nM) as indicated. After 12 hours treatment with Cpd E the cells were harvested, lysed with RIPA buffer and the lysates were separated by SDS-PAGE. Cleavage of endogenous APP was detected by immunoblotting with an anti-APP CTF antibody; expression of the PS1 constructs was confirmed by immunoblotting with 614.1 anti-PS1 NTF antibody. A β-actin blot was included as a loading control. Data are representative of experiments (n=3).

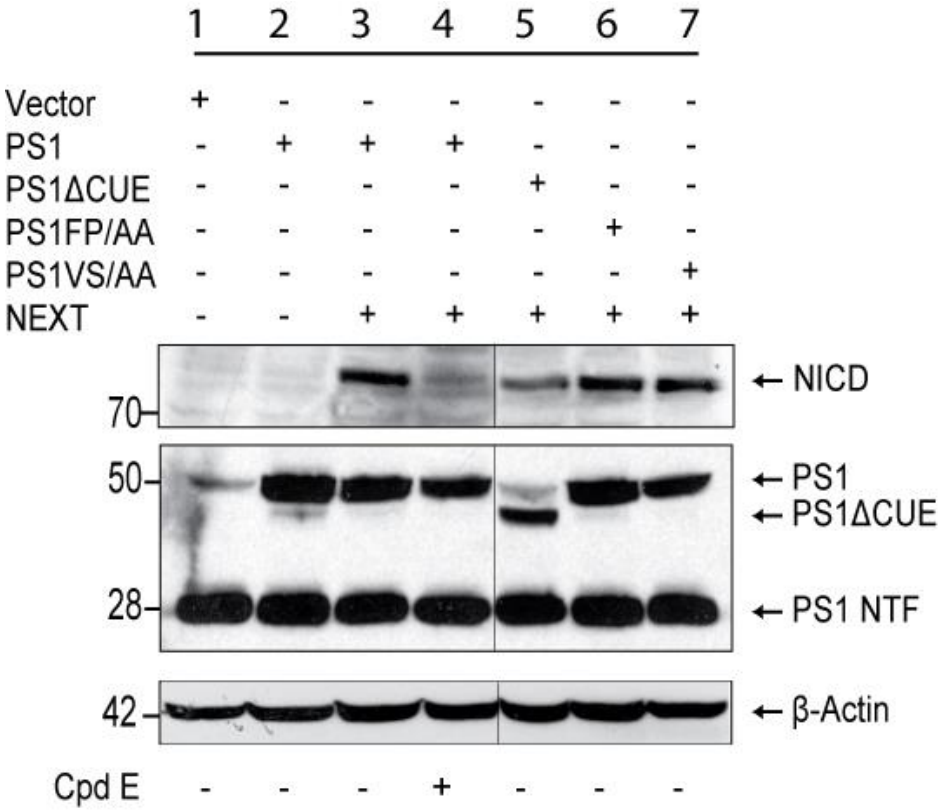


Figure 3.14 PS1 CUE domain function is dispensable for γ-secretase cleavage of Notch. HEK293T cells were transiently co-transfected with NEXT and pcDNA3-PS1 WT, pcDNA3-PS1 ΔCUE, pcDNA3-PS1 F283A/P284A or pcDNA3-PS1 V309A/S310A expression vectors as indicated. Twenty four hours after transfection cells were treated with the γ-secretase inhibitor compound E (Cpd E) (50 nM) as indicated. After 12 hours treatment with Cpd E the cells were harvested, lysed with RIPA buffer and the lysates were separated by SDS-PAGE. Cleavage of NEXT was detected by immunoblotting with an anti-cleaved Notch antibody; expression of the PS1 constructs was confirmed by immunoblotting with 614.1 anti-PS1 NTF antibody. A β-actin blot was included as a loading control. Data are representative of experiments (n=3).

polyubiquitin chains it was decided to investigate whether the PS1 CUE domain had any effect on the regulation of this γ-secretase substrate. HEK293T cells were transiently transfected with pcDNA3-IL-1R1 WT and with either pcDNA-PS1 WT, pcDNA-PS1 ΔCUE, pcDNA-PS1 F283A/P284A or pcDNA-PS1 V309A/S310A. To act as a negative control cells expressing both pcDNA-IL-1R1 and pcDNA-PS1 were pre-treated for 12 hours with γ-secretase inhibitor Compound E. To stimulate ectodomain shedding, cells were treated with PMA as indicated for 2 hours before harvesting. Treatment with PMA stimulates formation of the IL-1R1 ICD **(compare lanes 3 and 5 of Figure. 3.15)** in cells coexpressing pcDNA3-IL-1R1 and pcDNA3-PS1. However, as can be seen in lane 4 **(Figure 3.15)** that treatment with Cpd E inhibits formation of the IL-1R1 ICD fragment even when the cells are also treated with PMA. In cells coexpressing pcDNA3-IL-1R1 and the PS1 CUE mutants pcDNA-PS1 ΔCUE, pcDNA-PS1 F283A/P284A or pcDNA-PS1 V309A/S310A pre-treatment with PMA also caused the formation of the IL-1R1 ICD fragment **(see lanes 6-8 of Figure. 3.15)**. The level of IL-1R1 ICD was at a comparable level to the IL-1R1 ICD found in PMA treated cells expressing pcDNA3-PS1 WT (lane 5). This shows that deletion or mutation of the PS1 CUE domain has no effect on the γ-secretase mediated cleavage of IL-1R1.

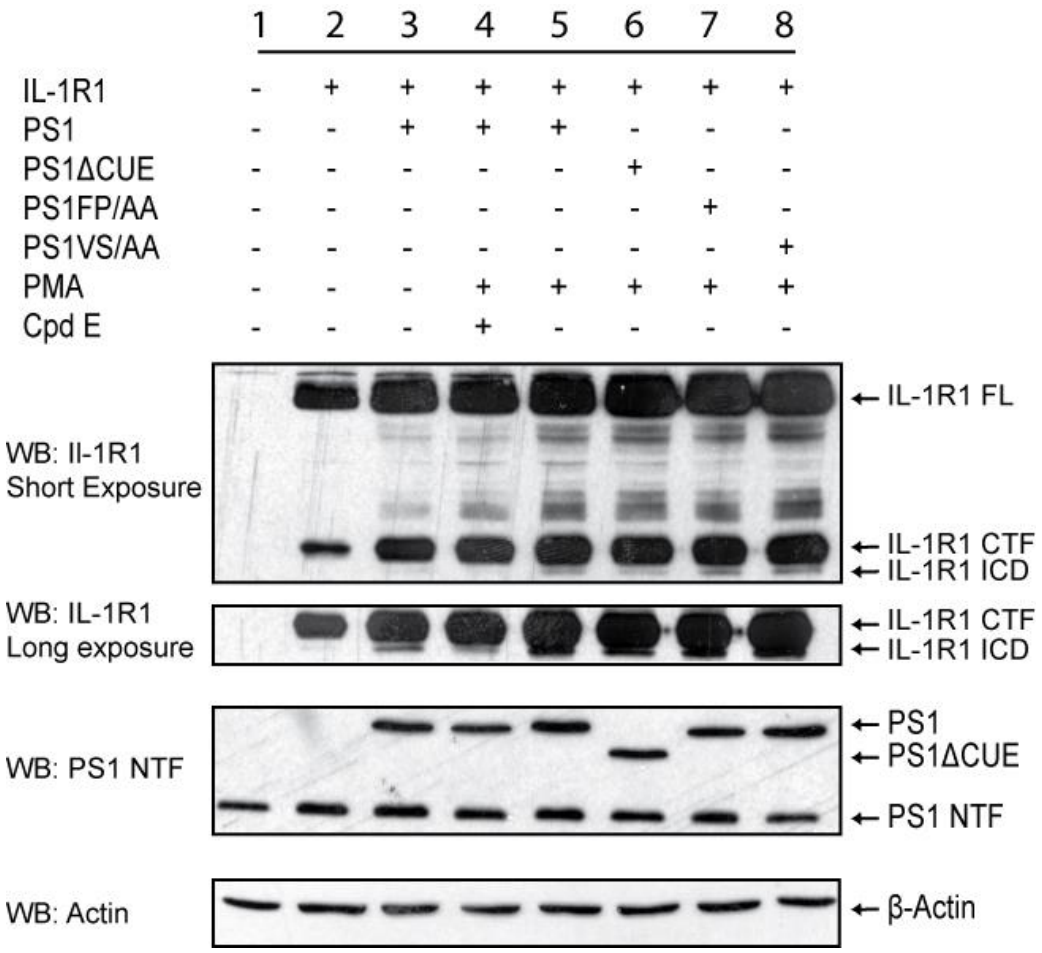


Figure 3.15 PS1 CUE domain function is dispensable for γ-secretase cleavage of IL-1R1. HEK293T cells were transiently co-transfected with pcDNA3-IL-1R1 and either pcDNA3-PS1 WT, pcDNA3-PS1 ΔCUE, pcDNA3-PS1 F283A/P284A or pcDNA3-PS1 V309A/S310A expression vectors. Twenty four hours after transfection cells were treated with the γ-secretase inhibitor compound E (Cpd E) (50 nM) as indicated. After 10 hours pre-treatment with Cpd E ectodomain shedding was induced by a 2 hr treatment with PMA (200 ng/ml). After harvesting, cells lysed with RIPA buffer and the lysates were separated by SDS-PAGE. Cleavage of IL-1R1 was detected by immunoblotting with an anti-IL-1R1 C20 antibody; expression of the PS1 constructs was confirmed by immunoblotting with 614.1 anti-PS1 NTF antibody. A β-actin blot was included as a loading control. Data are representative of experiments (n=2).

Discussion

The presenilin proteins are typically seen as the protease found at the heart of the γ-secretase complexes. In addition to that, the presenilin proteins are involved in a number of γ-secretase independent functions such as regulating calcium homeostasis [Tu *et al* 2006], β-catenin signalling [Soriano *et al* 2001], protein trafficking [Esselens *et al* 2004] and apoptosis [Zeng *et al* 2015]. The presenilin proteins undergo a number of post translational modifications, such as ubiquitination, phosphorylation and caspase cleavage, within the large intracellular loop domain that exists between TMDs 6 and 7 of both proteins (see Fig. 1.2). This intracellular loop has also been shown to be the site of protein-protein interactions that are involved in γ-secretase independent roles of the presenilins, e.g. β-catenin/δ-catenin and Rab11 [Zhou *et al* 1997, Dunmanchin *et al* 1999]. As such this intracellular loop domain represents an important region in investigating γ-secretase independent roles of presenilin proteins.

Previous work in our lab identified an area of homology between a region of the intracellular loop domains of the presenilins (residues 271-314 in human PS1 and 277-320 of human PS2) and the sequence of proteins containing an ubiquitin binding CUE domain **(Figure 3.1A)** [James Powell]. Both presenilins contained the highly conserved FP motif (residues F283/P284 for human PS1 and residues F289/P290 for human PS2) and a less well conserved di-leucine motif (residues V309/S310 for human PS1 and L315/Q316 for human PS2). These putative CUE domains in the presenilins were well conserved across different species and evolutionary time **(Figure 3.1B)**. To investigate the function of the presenilin CUE domains the CUE domain of both PS1 and PS2 were deleted, and the key FP and VS or LQ motifs were mutated in PS1 and PS2, respectively. Using an ubiquitin binding assay using

immunopurified presenilin proteins and recombinant polyubiquitin molecules it was shown that the presenilin CUE domains bind preferentially to K63-linked polyubiquitin chains over K48-linked polyubiquitin **(Figure 3.3 and 3.5)**. Additionally we have shown that the V309/S310 motif, but not the F283/P284 motif, is required for K63 polyubiquitin binding to the PS1 CUE domain **(Figure 3.4A)**. The effect of point mutations within the PS2 CUE domain remains to be explored **(Figure 3.5B)** To strengthen this observation a series of GST-tagged proteins containing the intracellular loop domain of PS1 were created **(Figure 3.7)**, which contained either the WT PS1 CUE domain or PS1 CUE domains with the conserved FP and VS motifs mutated either singly or in tandem. These recombinant proteins were then used in an ubiquitin pulldown assay to demonstrate PS1 CUE domain function. These experiments further demonstrate that the PS1 CUE domain preferentially binds to K63-linked polyubiquitin and that residues V309/S310 are required for this interaction **(Figure 3.10)**. Therefore, using both cell based and recombinant protein based approaches we have shown that PS1 contains a functional ubiquitin binding CUE domain within its intracellular loop domain and that the conserved V309/S310 motif is required for PS1 CUE domain function.

The Jnet secondary structure prediction software to analyse the structures of the putative CUE domains of the presenilins and those of known CUE domains **(Figure 3.1A)**. This analysis predicted a three helical structure of the examined CUE domains, where the conserved FP motif was found at the C-terminal end of the first helix and the semi-conserved di-leucine motif was found within the third helix. The structure of the CUE domains of Vps9 [Prag *et al* 2003] and Tollip [Azurmendi *et al* 2010] have been determined using X-ray crystallography and NMR, respectively. In the Vps9 CUE domain the FP motif directly interacts with the surface of ubiquitin

and the proline residue represents the end point of the first helix of the domain [Prag *et al* 2003]. However, in the Tollip CUE domain the first helix ends prior to the FP motif [Azurmendi *et al* 2010]. This discrepancy may help explain the reason why mutation of the F283/P284 motif of PS1 does not affect PS1 CUE domain function, that there is some variability in the structures of CUE domains. In Vps9 the di-leucine motif is present in the third helix of the CUE domain where the first leucine residue (L446) plays a role in stabilising the Vps9 CUE domain structure and the second lysine residue (L447) interacts with the surface of ubiquitin, including the K48 residue of ubiquitin [Prag *et al* 2003]. Applying this model to the PS1 CUE domain the PS1 V309 residue would be required for PS1 CUE domain secondary structure and the PS1 S310 residue would interact with the surface of ubiquitin proteins. This proposed interaction between S310 and ubiquitin may explain the importance of the V309/S310 motif to the binding of K63 polyubiquitin. This motif may represent the region that specifies the binding of K63 polyubiquitin to the PS1 CUE domain.

After showing that the presenilin CUE domains bind to K63-linked polyubiquitin it was decided to investigate whether the PS1 CUE domain had no role to play in more established presenilin functions. Firstly, it was shown that mutation of the F283/P284 and V309/S310 sites had no effect on PS1 endoproteolysis **(Figure 3.11)** in PS double knockout MEFs. Deletion of the PS1 CUE domain also deleted the site of PS1 endoproteolysis [Steiner *et al* 1999] so as expected this mutated protein does not undergo endoproteolysis. Next, the effect of PS1 CUE domain deletion or mutation of conserved CUE domain motifs on γ-secretase function was investigated using the PS1 ΔCUE, PS1 F283A/P284A and PS1 V309A/S310A mutants. The PS1 CUE domain was shown to be dispensable for APP cleavage in both HEK293T cells

(Figure 3.12A) and PS1 deficient MEFs **(Figure 3.13)**. Formation of the γ-secretase APP cleavage products Aβ40 and Aβ42 was also examined through ELISA, showing that CUE domain deletion or mutation of conserved motifs had no significant effect on levels of Aβ40/42 **(Figure 3.12B)**. Similarly the PS1 F283A/P284A and PS1 V309A/S310A mutations had no effect on cleavage of NEXT, a constitutively cleaved Notch construct, when co-expressed in HEK293T cells **(Figure 3.14)**. Finally, the γ-secretase cleavage of IL-1R1 was examined using these PS1 CUE domain mutants. Co-expression of the PS1 ΔCUE, PS1 F283A/P284A or PS1 V309A/S310A mutants with IL-1R1 WT had no effect on PMA induced IL-1R1 ICD formation in HEK293T **(Figure 3.15)**. These data demonstrate that mutation of the CUE domain of PS1 has no effect on either presenilin endoproteolysis or of the γ-secretase mediated cleavage of the γ-secretase substrates APP, Notch and IL-1R1.

However, when the PS1 ΔCUE mutant was co-expressed with the NEXT construct in HEK293T cells there was a reduction in NICD formation was compared to PS1 WT or to the PS1 F283A/P284A or PS1 V309A/S310A CUE domain point mutants **(Figure 3.14)**. Additionally, there was a reduction in formation of both Aβ40 and Aβ42 in HEK293T cells overexpressing the PS1 ΔCUE mutant, though this reduction was not found to be statistically significant. These data raise the possibility that the non-endoproteolytically cleaved PS1 ΔCUE mutant may have a reduced γ-secretase function compared to PS1 proteins which undergo endoproteolysis. The PS1 ΔE9 mutation causes the formation of a non-cleavable PS1 protein, missing residues 290-319 of PS1, which contains the site of endoproteolysis [Rovelet-Lecrux *et al* 2015]. Expression of the PS1 ΔE9 mutant in presenilin null MEFs has been shown to cause a reduction in the formation of AICD and Aβ40 and Aβ42 as compared to expression of PS1 WT [Cacquevel *et al* 2012]. Neurons formed from

induced pluripotent stem cells expressing the PS1 ΔE9 mutation show a greater expression of APP CTF while also showing an increase in the Aβ42 to Aβ40 ratio. These data suggest that the formation of a non-endoproteolytically cleavable PS1 holoprotein can have an effect on γ-secretase function within cells. This may suggest a reason for the differences in γ-secretase function within cells expressing the PS1 ΔCUE mutant.

The intracellular loop domains of the presenilins represent a region with numerous protein-protein interactions [McCarthy *et al* 2009] and post translational modifications **[see Figure 1.2]**. The presence of ubiquitin binding CUE domains in the presenilin proteins therefore represents a novel way in which presenilin function can be regulated. The serine residue of the conserved V309/S310 motif of the PS1 CUE domain has previously been shown to be a site of PKA phosphorylation [Fluhrer *et al* 2004]. To investigate whether PKA phosphorylation regulates PS1 CUE domain function a pseudo-phosphorylated PS1 S310E mutant was created and used in an ubiquitin binding assay **(Figure 3.4)**. This pseudo-phosphorylated PS1 S310E mutant showed no difference in K63-linked polyubiquitin binding compared to PS1 WT, suggesting that PS1 CUE domain function is not regulated by PKA phosphorylation.

The CUE domain of the presenilins spans the sites of endoproteolysis, suggesting that the presenilin CUE domains are only functional in the immature holoproteins. Having shown that the presenilins can interact with K63-linked polyubiquitin chains, next we need to explain the function of this novel presenilin domain. While K48-linked polyubiquitination is primarily associated with protein degradation via the ubiquitin proteasome system, K63 ubiquitination is known to be involved in a number of other pathways including DNA repair and cell signalling pathways [Ge *et*

al 2015, Wang *et al* 2015]. The presenilins are known to interact with over 50 proteins that cover a number of cellular pathways and functions [reviewed in McCarthy *et al* 2009]. The presenilins are also known to be involved in a number of γ-secretase independent roles, including those that are associated with the holoprotein. In the next chapter we will begin to explore the function of the PS1 CUE domain by investigating the effect of the PS1 CUE domain on a number of PS1 related protein-protein interactions.

4 PS1 Interacting Proteins and the PS1 CUE Domain

Introduction

In chapter 3 it was demonstrated that the presenilins contain functional CUE domains that selectively bind to K63-linked polyubiquitin chains. It was further demonstrated that the PS1 CUE domain is dispensable for PS1 endoproteolysis and the γ-secretase mediated cleavage of APP, Notch and IL-1R1. Having shown that PS1 contains an ubiquitin binding CUE domain within its intracellular loop domain, the possible function(s) of this domain was next considered. We hypothesised that the PS1 CUE domain is involved in regulating intermolecular or intramolecular presenilin protein-protein interactions. By this we mean that the PS1 CUE domain could be involved in interacting with K63-linked polyubiquitinated PS1 interacting proteins or the PS1 CUE domain interacts with K63-linked polyubiquitinated PS1 proteins. Therefore, we reasoned that through the identification of the PS1 CUE domain interacting protein(s) it would be possible to begin to elucidate the function(s) of this novel ubiquitin binding domain within PS1.

The presenilins were first discovered through research into genetic linkages in familial Alzheimer's disease and much early research was focused on discovering presenilin interacting proteins as a means of investigating presenilin function. The γ-secretase protease complex is comprised of presenilins, Nicastrin [Yu *et al* 2000], Aph1 and Pen-2 [Hebert *et al* 2004]. While the identity of the members of the γ-secretase complex had been determined, further research into the presenilins demonstrated that the presenilins had a number of γ-secretase independent functions such as involvement in calcium homeostasis [Tu *et al* 2006], apoptosis [Guo *et al* 1996] and protein trafficking [Naruse *et al* 1998]. As such identifying presenilin

interacting proteins remained a research priority, leading to the discovery of an ever increasing number of presenilin interacting proteins such as the catenins [Zhou *et al* 1997], GSK3β [Takashima *et al* 1998] and calsenilin [Buxbaum *et al* 1998] amongst many others [McCarthy *et al* 2009; Soler-Lopez *et al* 2011]. The involvement of the presenilins in diverse cell signalling pathways has been shown through the identification of presenilin interacting proteins. For example, expression of PS1 and PS2 FAD mutants was observed to lead to an increase in apoptosis [Guo *et al* 1997; Janicki *et al* 1997] which lead to the discovery that the presenilins interact directly with the apoptotic proteins such as Bcl-2 [Alberici *et al* 1999], Bcl-2L1 [Passer *et al* 1999] or the presenilin-associated protein (PSAP) [Xu *et al* 2002]. Conversely identification of presenilin interacting proteins has also suggested novel signalling for the presenilins, e.g. once the presenilins were shown to interact with the catenin proteins [Zhou *et al* 1997] it lead to the discovery that the presenilin were involved in the regulation of Wnt signalling [Kawamura *et al* 1998] and β-catenin mediated apoptosis [Zhang *et al* 2008].

Apart from the proteins that make up the γ-secretase complex, PS1 and PS2 share a number of common interacting proteins including the γ-secretase substrate APP, glutaryl-CoA dehydrogenase (GCDH), the E3 ligase F-box/LRR-repeat protein 12 (FBXL12), the adaptor protein ECSIT and programmed cell death 4 (PDCD4) have all been shown to directly interact with both PS1 and PS2 [Soler-Lopez *et al* 2011]. Calsenilin [Buxbaum *et al* 1998] calmyrin [Stabler *et al* 1999], KCNIP4 [Morohashi *et al* 2002] and the calcium channels SERCA [Green *et al* 2008] and InsP3R [Cai *et al* 2006; Cheung *et al* 2008], which all interact with both presenilins, are all involved in the regulation of calcium homeostasis. This supports the observation that both PS1 and PS2 are involved in calcium regulation [Muller *et al* 2011; Kipanyula *et al*

2012]. The adhesion protein ICAM5 [Annaert *et al* 2001], the glial fibrillary acidic protein (GFAP) [Nielsen *et al* 2002] and the cytoskeleton protein filamin B [Zhang *et al* 1998] are structural proteins that also interact with both proteins. The armadillo proteins β-catenin and δ-catenin, which associate with the cadherin adhesion proteins, interact with the intracellular loops of PS1 and PS2 [Levesque *et al* 1999]. FAD mutations within both presenilin proteins inhibit the translocation of β-catenin to the nucleus [Nishimura *et al* 1999] suggesting that both presenilin proteins are involved in the regulation of β-catenin signalling. The E3 ligase TRAF6 interacts and ubiquitinates both presenilin proteins [Elzinga *et al* 2009; Yan *et al* 2013] and the UBA domain containing protein ubiquilin also interacts with PS1 and PS2 [Mah *et al* 2000]. The Bcl-2 family member Bcl-2L1 interacts with both presenilins [Passer *et al* 1999] while only PS1 has been shown to interact with Bcl-2 [Alberici *et al* 1999]. Similarly, PSAP interacts solely with PS1 [Xu *et al* 2002] and upregulates apoptosis through its interaction with Bax [Zeng *et al* 2015]. These differences in protein-protein interactions between the presenilin proteins may explain the differences in apoptosis observed between PS1 and PS2 [Amson *et al* 2000; Alves da Costa *et al* 2003].

CUE domains have been shown to be involved in a number of pathways such as autophagy [Lu *et al* 2014], protein turnover [Bagola *et al* 2013], endocytosis [Davies *et al* 2014] and cell signalling [Kishida *et al* 2005]. CUE domains have also been shown to be involved in the regulation of proteins turnover and stability within the cell in a number of discrete ways. The yeast protein Cue5 and its mammalian homolog Tollip are involved in the autophagic turnover of Poly-Q proteins, such as huntingtin [Lu *et al* 2014]. Similarly the CUE domain of Cue1p is involved in regulating the turnover of proteins through ERAD [Bagola *et al* 2013. Conversely,

the CUE domain of FANCD2 prevents the polyubiquitination and proteasomal degradation of FANCD2 through its binding to monoubiquitin [Rego *et al* 2012]. The CUE domain of Vps9 has been shown to be essential for endocytosis in yeast cells [Davies *et al* 2003]. CUE domains have also been shown to be involved in the regulation of cell signalling pathways. The CUE domain of TAB2 is required for the ubiquitination of TRAF6 and for the propagation of IL-1 signalling in human 293 cells [Kishida *et al* 2005] while the CUE domain of CUEDC2 is involved regulating progesterone receptor signalling [Zhang *et al* 2007] and mitotic regulation [Gao *et al* 2011]. Given that the presenilins and ubiquitin binding CUE domains can be involved in multiple cellular functions and pathways, it was decided to first focus the search for PS1 CUE domain interacting proteins on known PS1 interacting proteins that undergo K63-linked polyubiquitination.

In this chapter a list of published presenilin-interacting proteins are compiled and their published ubiquitination states are identified. This list is then narrowed down to include K63-linked polyubiquitinated proteins which also interact with PS1. Then using a bioinformatics based approach the sites of potential ubiquitination within these proteins are identified. The TRAFs are a family of E3 RING domain containing proteins that are involved regulating cell signalling pathways [Silke and Melino 2009]. TRAF6 has previously been shown to promote the K63-linked polyubiquitination of PS1 [Yan *et al* 2013; Gudey *et al* 2014]. In this study, using co-immunoprecipitation assays the E3 ligase activity of TRA6 was shown to be required for the interaction between TRAF6 and PS1. The PS1 CUE domain was also shown to be dispensable for this interaction with TRAF6, but mutation of conserved residues within the PS1 CUE domain was shown to be required for the interaction between PS1 and TRAF6. However, mutation of these conserved PS1

CUE domain residues had no effect on the TRAF6 mediated increased stability of the PS1 holoprotein. Additionally, previous studies in our lab have shown that PS1 interacts with TRAF2 [Powell unpublished]. In this study, using an ubiquitination assay we showed that PS1 is a target for TRAF2 mediated K63-linked polyubiquitination but the PS1 CUE domain is dispensable for this interaction between PS1 and TRAF2.

4.1 Presenilin Interacting Proteins

In addition to their interactions with Aph1, nicastrin and Pen-2 as part of the γ-secretase complex the presenilins are known to interact with a large number of additional proteins [McCarthy *et al* 2009; Soler-Lopez *et al* 2011]. As a way of investigating the function of the PS1 CUE domain it was decided to analyse known PS1 interacting proteins to look for proteins which are subject to K63-linked polyubiquitination. Any PS1 interacting protein that is subject to K63-linked polyubiquitination could represent a protein that interacts with the K63-linked polyubiquitin binding PS1 CUE domain. To construct the PS1 interactome a list of PS1 interacting proteins was compiled from conflating a previously published source [McCarthy *et al* 2009] and an online database of published human PS1 interacting proteins obtained from the BioGRID [http://thebiogrid.org/111642/summary/homo-sapiens/psen1.html]. The BioGRID (http://thebiogrid.org/) is an online database that records published protein-protein interactions and known sites of posttranslational modification. Therefore, once the list of PS1 interacting proteins was generated the potential for ubiquitination of these proteins was investigated using the BioGRID. These data were compiled **(Table 4.1)** providing the gene symbol, protein name, type of ubiquitination and citations for both the interaction with PS1 and for the proteins ubiquitination. For many of these proteins, for example CD147 [Beltrao *et al* 2012], FKBP38, GDI1 [Udeshi *et al* 2013] etc., their ubiquitination was determined using high throughput screening using mass spectrometry. Using this high throughput approach CD147 was shown to be ubiquitinated at 13 different lysine residues [Beltrao *et al* 2012], FKBP38 at 9 different lysine residues and GDI1 at 12 lysine residues [Udeshi *et al* 2013]. For these proteins the high throughput analysis would give the site of ubiquitination but would not be able to determine

whether this was a site of monoubiquitin or polyubiquitination nor would it provide the type of polyubiquitin chains that could be bound to that site. For all proteins whose ubiquitination was determined in this manner the type of ubiquitination is recorded as 'screen'. For some proteins that undergo ubiquitination the type of ubiquitination is determined through ubiquitination assays but the type of polyubiquitination is not determined so these are recorded as 'not specified'. In other cases the type of ubiquitination is not determined but the proteins ubiquitination is involved in determining protein turnover so these are recorded as 'not specified UPS'. If the type of protein ubiquitination is known it is recorded e.g. mono, K48, K63, etc.

From the data provided in **(Table 4.1)**, all reported PS1 interacting protein that are known to undergo K63-linked polyubiquitination are summarized in **(Table 4.2)**. This table provides the gene symbol, protein name and a citation for the K63-linked polyubiquitination of these PS1 interacting proteins. The proteins **(Table 4.2)** provided a start-point from which it was possible to begin investigating the function(s) of the PS1 CUE domain.

These K63-linked polyubiquitinated PS1-interacting proteins are involved in a variety of cell signalling pathways. BACE and Tau are proteins that are associated with the β-amyloid and neurofibrillary tangle pathologies of AD, respectively [Zou *et al* 2014]. Meanwhile, Fe65 is a protein that interacts with the AICD to regulate gene transcription [Vazquez *et al* 2009]. BACE1 is ubiquitinated at residue K501 by either monoubiquitin or K63-linked polyubiquitination [Kang *et al* 2008]. Fe65 turnover is regulated by its K63-linked polyubiquitination by the E3 ligase RNF157 [Matz *et al* 2015] and similarly K63-linked polyubiquitination of Tau causes the p62-mediated proteasomal degradation of the protein [Babu *et al* 2005]. Best

Table 4.1 Presenilin-1 Interacting Proteins and their known ubiquitination type

Protein name	Protein Name	Type	Interaction citation	Ubiquitination Citation
ACHE	acetylcholinesterase		Silveyra et al 2008	
ACTN1	actinin, alpha 1	screen	Soler-Lopez et al 2011	Povlsen et al 2012
AP1M2	adaptor-related protein complex 1, mu 2 subunit	screen	Soler-Lopez et al 2011	Beltrao et al 2012
APBA1	amyloid beta (A4) precursor protein-binding, family A		Lau et al 2000	
APBB1	amyloid beta (A4) precursor protein-binding, family B	K63	Lau et al 2000	Matz et al 2015
APH1A	APH1A gamma secretase subunit	screen	Hebert et al 2004	Beltrao et al 2012
APH1B	APH1B gamma secretase subunit	screen	Hebert et al 2004	Beltrao et al 2012
APOE	apolipoprotein E	not specified	Soler-Lopez et al 2011	Wenner et al 2001
APP	amyloid beta (A4) precursor protein	K48	Soler-Lopez et al 2011	Beltrao et al 2012
BACE	BACE	mono/k63	Hebert et al 2003	Kang et al 2008
BCL2	B-cell CLL/lymphoma 2	mono	Alberici et al 1999	Chen et al 2010
BCL2L1	BCL2-like 1	K48	Passer et al 1999	Wagner et al 2011
CAPN1	Calpain		Shinozaki et al 1998	Beltrao et al 2012
CD147	CD147		Zhou et al 2005	Beltrao et al 2012
CDC37	cell division cycle 37	screen	Soler-Lopez et al 2011	Beltrao et al 2012
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	K48	Georgakopoulos et al 1999	Yang et al 2006
CDK5	cyclin-dependent kinase 5	K48	Soler-Lopez et al 2011	Udeshi et al 2013
CFL1	cofilin 1 (non-muscle)	screen	Soler-Lopez et al 2011	Danielsen et al 2011
CIB1	calcium and integrin binding 1 (calmyrin)	screen	Stabler et al 1999	Kim et al 2011
CLIP1	CLIP1/CLIP170	screen	Johnsingh et al 2000	Stes et al 2014
CLSTN1	calsyntenin 1	screen	Araki et al 2004	Beltrao et al 2012
CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa	screen	Georgakopoulos et al 1999	Povlsen et al 2012
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	K11, K48, K63	Raurell et al 2006	Dao et al 2012
CTNND1	catenin (cadherin-associated protein), delta 1	screen	Tanahashi et al 1999	Kim et al 2011
CYP2C18	cytochrome P450, family 2, subfamily C, polypeptide 18		Soler-Lopez et al 2011	
CYP2C8	cytochrome P450, family 2, subfamily C, polypeptide 8		Soler-Lopez et al 2011	
DHX9	DEAH (Asp-Glu-Ala-His) box helicase 9	screen	Havugimama et al 2012	Povlsen et al 2012

DOCK3	dedicator of cytokinesis 3	screen	Kashiwa et al 2000	Danielsen et al 2011
DSP	desmoplakin	screen	Raurell et al 2006	Beltrao et al 2012
DTNA	dystrobrevin, alpha		Havugimama et al 2012	
ECSIT	ECSIT signalling integrator	k63	Soler-Lopez et al 2011	Mi Wi et al 2015
EFHD1	EF-hand domain family, member D1	screen	Soler-Lopez et al 2011	Oshikawa et al 2012
ENSA	endosulfine alpha	screen	Soler-Lopez et al 2011	Stes et al 2014
EPB41L3	erythrocyte membrane protein band 4.1-like 3	screen	Soler-Lopez et al 2011	Povlsen et al 2012
ERLIN2	ER lipid raft associated 2	screen	Teranishi et al 2012	Beltrao et al 2012
ERN1	endoplasmic reticulum to nucleus signaling 1	K63	Katayama et al 1999	Zhu et al 2014
ETFA	electron-transfer-flavoprotein, alpha polypeptide	screen	Van Gassen et al 1999	Beltrao et al 2012
FBXL12	F-box and leucine-rich repeat protein 12	screen	Soler-Lopez et al 2011	Beltrao et al 2012
FBXW7	F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase		Li et al 2002	Udeshi et al 2013
FGF13	fibroblast growth factor 13		Soler-Lopez et al 2011	
FKBP38	FKBP38	screen	Wang et al 2005	Povlsen et al 2012
FLNB	filamin B, beta	screen	Zhang et al 1998	Udeshi et al 2013
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	screen	Soler-Lopez et al 2011	Kim et al 2011
GCDH	glutaryl-CoA dehydrogenase	screen	Soler-Lopez et al 2011	Udeshi et al 2013
GDI1	GDP dissociation inhibitor 1	screen	Scheper et al 2000	Udeshi et al 2013
GFAP	glial fibrillary acidic protein	not specified	Nielsen et al 2002	Beltrao et al 2012
GNAO1	guanine nucleotide binding protein (G protein), alpha	screen	Smine et al 1998	Udeshi et al 2013
GRB2	growth factor receptor bound protein 2	screen	Nizzari et al 2007	Beltrao et al 2012
GRIN1	glutamate receptor, ionotropic, NMDA1 (zeta 1)		Saura et al 2004	
GSAP	gamma-secretase activating protein		He et al 2010	
GSK3B	glycogen synthase kinase 3 beta	mono/not specified	Takashima et al 1998	Gao et al 2014
HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	screen	Sai et al 2002	Beltrao et al 2012
HMGB1	high mobility group box 1	screen	Soler-Lopez et al 2011	Beltrao et al 2012
HTRA2	HtrA serine peptidase 2	screen	Gupta et al 2004	Beltrao et al 2012
ICAM5	intercellular adhesion molecule 5, telencephalin		Annaert et al 2001	
IRAK2	interleukin-1 receptor-associated kinase 2	screen	Elzinga et al 2009	Lee et al 2011

ITPR1	inositol 1,4,5-trisphosphate receptor, type 1	not specified UPS	Cheung et al 2008	Sarraf et al 2013
ITSN2	intersectin 2	screen	Soler-Lopez et al 2011	Stes et al 2014
JUP	junction plakoglobin	screen	Raurell et al 2006	Beltrao et al 2012
KANK2	KN motif and ankyrin repeat domains 2	screen	Soler-Lopez et al 2011	Kim et al 2011
KCNIP3	Calsenilin	not specified UPS	Buxbaum et al 1998	Jang et al 2011
KCNIP4	Kv channel interacting protein 4		Morohashi et al 2002	
MAPT	microtubule-associated protein tau	K63	Takashima et al 1998	Babu et al 2005
MET1	Met1 methyltransferase		Zhang et al 2001	
NCSTN	nicastrin	screen	Yu et al 2000	Udeshi et al 2013
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	screen	Havugimana et al 2012	Beltrao et al 2012
NOS3	nitric oxide synthase 3 (endothelial cell)	screen	Soler-Lopez et al 2011	Beltrao et al 2012
NOTCH1	notch 1	k29/mono	Ray et al 1999	Chastagner et al 2008
OXCT1	3-oxoacid CoA transferase 1	screen	Soler-Lopez et al 2011	Stes et al 2014
PARL	presenilin associated, rhomboid-like	screen	Pellegrini et al 2001	Beltrao et al 2012
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	not specified UPS	Soler-Lopez et al 2011	Beltrao et al 2012
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)		Baki et al 2004	
PKP4	plakophilin 4	screen	Stahl et al 1999	Beltrao et al 2012
PLD1	phospholipase D1	mono-multi	Cai et al 2006	Yin et al 2010
PRAM1	PML-RARA regulated adaptor molecule 1		Soler-lopez et al 2011	
PRDX1	Peroxiredoxin 1 PAG	k48	Zhou et al 2002	Nasu et al 2010
PRDX2	peroxiredoxin 2	screen	Soler-Lopez et al 2011	Beltrao et al 2012
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	screen	Kang et al 2002	Beltrao et al 2012
PSEN1	presenilin 1	K63	Hebert et al 2004	Yan et al 2013
PSEN2	presenilin 2	K63	Hebert et al 2004	Yan et al 2013
PSENEN	presenilin enhancer gamma secretase subunit		Hebert et al 2004	
PSMA5	proteasome (prosome, macropain) subunit, alpha type, 5	screen	Van Gassen et al 1999	Beltrao et al 2012
PSMB1	proteasome (prosome, macropain) subunit, beta type, 1	screen	Van Gassen et al 1999	Beltrao et al 2012
RAB11A	RAB11A	screen	Dumanchin et al 1999	Udeshi et al 2013
RAB3A	RAB3A, member RAS oncogene family		Soler-lopez et al 2011	
RAD23A	RAD23 homolog A (S. cerevisiae)		Soler-Lopez et al 2011	
RHEB	Ras homolog enriched in brain	screen	Soler-Lopez et al 2011	Beltrao et al 2012
RIPK1	receptor (TNFRSF)-interacting	K63	Unpublished data	Ea et al 2006

	serine-threonine kinase 1			
RMDN3	regulator of microtubule dynamics 3	screen	Soler-Lopez et al 2011	Danielsen et al 2011
RNF32	ring finger protein 32		Soler-Lopez et al 2011	
RYR2	ryanodine receptor 2	screen	Takeda et al 2005	Sarraf et al 2013
SCN1A	sodium channel, voltage-gated, type I, alpha subunit		Soler-Lopez et al 2011	
SRI	Sorcin	screen	Pack-Chung et al 2000	Stes et al 2014
ST13	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	screen	Soler-Lopez et al 2011	Beltrao et al 2012
STAMBPL1	STAM binding protein-like 1	screen	Soler-Lopez et al 2011	Udeshi et al 2013
STX1A	Syntaxin 1A		smith et al 2000	
STX5	Syntaxin 5	screen	Suga et al 2004	Povlsen et al 2012
TCF4	transcription factor 4	not specified	Raurell et al 2006	Yamada et al 2006
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	screen	Palacino et al 2001	Udeshi et al 2013
TDP2	tyrosyl-DNA phosphodiesterase 2	screen	Wang et al 2011	Danielsen et al 2011
TGFBR1	transforming growth factor, beta receptor 1	K63	Gudey et al 2014	Mu et al 2011
TMED10	TMP21	screen	Chen et al 2006	Beltrao et al 2012
TPTE2	transmembrane phosphoinositide -phosphatase and tensin homolog 2		Prihar et al 1999	
TRAF2	TNF receptor-associated factor 2, E3 ubiquitin protein ligase	K63	Unpublished data	Li et al 2009
TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase	k63	Elzinga et al 2009	Wooten et al 2005
TUBA1B	tubulin, alpha 1b	screen	Soler-Lopez et al 2011	Danielsen et al 2011
UBC	ubiquitin C		Yan et al 2013	Danielsen et al 2011
UBQLN1	ubiquilin 1		Mah et al 2000	Beltrao et al 2012
UMPS	uridine monophosphate synthetase	screen	Soler-Lopez et al 2011	Stes et al 2014
YME1L1	YME1-like 1 ATPase	screen	Pellegrini et al 2001	Kim et al 2011

characterised as a signalling molecule that links transmembrane adhesion proteins with Wnt signalling and gene transcription [Rosenbluh *et al* 2014], β-catenin is regulated by K11- and K63-linked polyubiquitination by FANCL [Dao *et al* 2012]. ECSIT, RIP1, TRAF2 and TRAF6 are adaptor proteins that are involved in signal transduction [Wi *et al* 2014; De *et al* 2014; Park *et al* 2015; Lamothe *et al* 2007]. The K63-linked polyubiquitin of ECSIT at K372 [Mi Wi *et al* 2015] and RIP1 at

K377 [Ea *et al* 2006] are required for ECSIT- and RIP1-mediated NFκB activation, respectively. TRAF2 is K63-linked polyubiquitinated at residue K31, which regulates TRAF2 binding to TAB2/3, and is required for TRAF2 activation of IKK and JNK [Li *et al* 2009]. TRAF6 undergoes K63-linked autoubiquitination at K124 and ubiquitination at this site is required for NFκB signalling [Lamothe *et al* 2007]. ERN1/IRE1 is involved in monitoring the unfolded protein response in the ER; IRE1 is K63-linked polyubiquitinated at residues K545 and K828. K63-linked polyubiquitination at K828 was shown to be essential for the interaction between IRE1 and TRAF2 under stress conditions [Zhu *et al* 2014].

Previous work in our lab has identified RIP1, TRAF2 and TRAF6 as PS1 interactng proteins. While RIP1 was shown to interact with PS1, the site of the interaction has not been mapped [Frances Harte unpublished work]. Therefore the PS1 CUE domain mutants will be used to determine whether the PS1 CUE domain is required for the interaction between RIP1 and PS1. The major interaction site for TRAF2 (P/S/T/A)X(Q/E)E has been identified in the intracellular loops of both PS1 and PS2 [James Powell unpublished work]. Additionally a TRAF6 interaction motif PxExxAr/Ac hass been mapped to the intracellular loop of PS1 [Powell *et al* 2009]. The TRAF2 and TRAF6 interaction sites are shown in **Figure 4.1**. Thus, both TRAF2 and TRAF6 are known to undergo K63-linked polyubiquitination and interact with PS1 within the intracellular loop domain and represent strong targets for investigating possible PS1 CUE domain interacting proteins.

4.2 Ubiquitination Site Prediction for Presenilin-1 Interacting Proteins

Using bioinformatic tools, an analysis of the potential for ubiquitination amongst the list of known PS1 interacting proteins **(Table 4.1)** was also conducted. An

ubiquitination prediction tool called Ubpred (www.ubpred.org) was used for this purpose. Ubpred analyses the primary structure of submitted proteins and determines the likelihood of any lysine residues to be sites of ubiquitination. These sites are scored on a scale of 0 to 1 where 1 is the highest likelihood for a lysine residue to be a site of ubiquitination. A residue with an Ubpred score of 0.62-0.69 is given a low confidence rating as an ubiquitination site, while scores of 0.69-0.84 and 0.84-1 are given medium confidence and high confidence ratings, respectively. The primary sequences of the PS1 interacting proteins **(Table 4.1)** were analysed by Upred and the number of potential ubiquitination sites was split into low, medium and high confidence ratings. These data were compiled **(Table 4.3)** providing the gene symbol, protein name and number of potential ubiquitination sites for each protein examined.

Table 4.2 PS-1 interacting proteins that undergo K63-linked polyubiquitination

Gene	Protein	Citation
APBB1	amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	Matz et al 2015
BACE	BACE	Kang et al 2008
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	Dao et al 2012
ECSIT	ECSIT signalling integrator	Mi Wi et al 2015
ERN1	endoplasmic reticulum to nucleus signaling 1	Zhu et al 2014
MAPT	microtubule-associated protein tau	Babu et al 2005
RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1	Ea et al 2006
TGFBR1	transforming growth factor, beta receptor 1	Mu et al 2011
TRAF2	TNF receptor-associated factor 2, E3 ubiquitin protein ligase	Li et al 2009
TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase	Wooten et al 2005

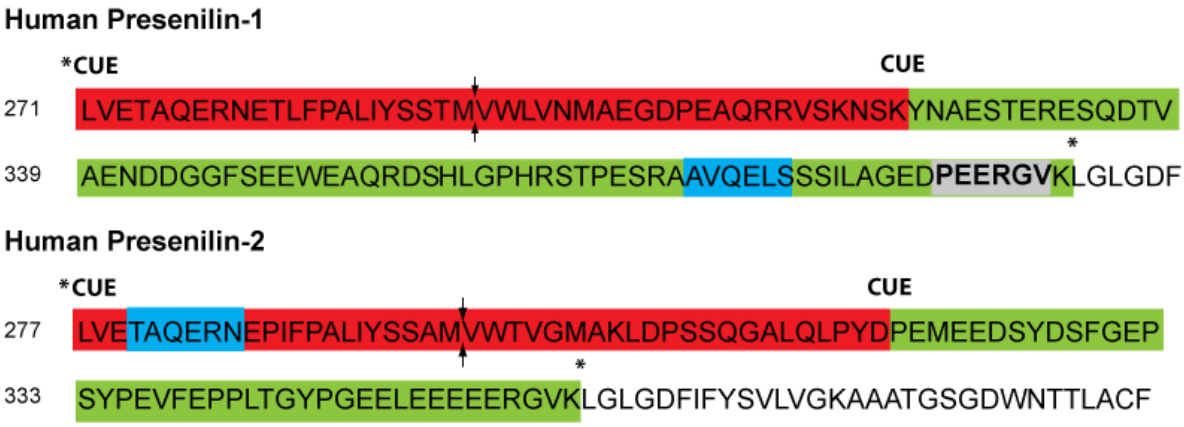


Figure 4.1 Schematic of Presenilin Intracellular Loop Domains including TRAF2 and TRAF6 Interaction Sites. Schematic shows the CUE domains of PS1 and PS2 (highlighted in red and bookended by 'CUE') and the remainder of the intracellular loop domains of the presenilins (highlighted in green and bookended by the symbol *). The sites of presenilin endoproteolysis are marked by arrows. The TRAF2 interaction sites (highlighted in blue) and the TRAF6 interaction site (highlighted in grey) are also shown.

Table 4.3 Ubpred predicted ubiquitination sites in PS-1 interacting proteins

gene	Protein Name	Number of Potential Ubiquitination Sites		
		low	medium	high
ACHE	acetylcholinesterase	0	1	0
ACTN1	actinin, alpha 1	4	1	0
AP1M2	adaptor-related protein complex 1, mu 2 subunit	2	3	0
APBA1	amyloid beta (A4) precursor protein-binding, family A	1	7	5
APBB1	amyloid beta (A4) precursor protein-binding, family B	3	6	2
APH1A	APH1A gamma secretase subunit	0	0	0
APH1B	APH1B gamma secretase subunit	1	0	0
APOE	apolipoprotein E	1	3	3
APP	amyloid beta (A4) precursor protein	3	8	5
BACE	BACE	0	3	0
BCL2	B-cell CLL/lymphoma 2	0	0	0
BCL2L1	BCL2-like 1	0	0	0
CAPN1	Calpain	1	0	0
CD147	CD147	2	3	0
CDC37	cell division cycle 37	7	4	11
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	5	0	1
CDK5	cyclin-dependent kinase 5	1	0	0
CFL1	cofilin 1 (non-muscle)	2	2	0
CIB1	calcium and integrin binding 1 (calmyrin)	0	1	0
CLIP1	CLIP1/CLIP170	20	58	42
CLSTN1	calsyntenin 1	3	4	0
CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa	4	9	0
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	4	2	0
CTNND1	catenin (cadherin-associated protein), delta 1	7	5	3
CYP2C18	cytochrome P450, family 2, subfamily C, polypeptide 18	0	1	0
CYP2C8	cytochrome P450, family 2, subfamily C, polypeptide 8	1	0	0
DHX9	DEAH (Asp-Glu-Ala-His) box helicase 9	4	4	1

DOCK3	dedicator of cytokinesis 3	14	10	1
DSP	desmoplakin	40	68	16
DTNA	dystrobrevin, alpha	2	8	4
ECSIT	ECSIT signalling integrator	0	1	1
EFHD1	EF-hand domain family, member D1	1	5	0
ENSA	endosulfine alpha	0	3	1
EPB41L3	erythrocyte membrane protein band 4.1-like 3	5	17	16
ERLIN2	ER lipid raft associated 2	6	2	1
ERN1	endoplasmic reticulum to nucleus signaling 1	5	9	1
ETFA	electron-transfer-flavoprotein, alpha polypeptide	1	2	0
FBXL12	F-box and leucine-rich repeat protein 12	0	0	0
FBXW7	F-box and WD repeat domain containing 7	1	0	0
FGF13	fibroblast growth factor 13	2	1	1
FHL2	FHL2/DRAL/SLIM-3	0	0	0
FKBP38	FKBP38	0	1	0
FLNB	filamin B, beta	19	16	0
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	1	0	0
GCDH	glutaryl-CoA dehydrogenase	1	2	0
GDI1	GDP dissociation inhibitor 1	0	3	0
GFAP	glial fibrillary acidic protein	4	2	0
GNAO1	guanine nucleotide binding protein (G protein)	3	2	0
GRB2	growth factor receptor bound protein 2	0	0	0
GRIN1	glutamate receptor, ionotropic, NMDA1 (zeta 1)	2	3	0
GSAP	gamma-secretase activating protein	0	0	0
GSK3B	glycogen synthase kinase 3 beta	0	3	0
HERPUD 1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	0	2	1
HMGB1	high mobility group box 1	2	3	0
HTRA2	HtrA serine peptidase 2	0	0	0
ICAM5	intercellular adhesion molecule 5, telencephalin	0	0	0
IRAK2	interleukin-1 receptor-associated kinase 2	2	1	5

ITPR1	inositol 1,4,5-trisphosphate receptor, type 1	17	19	4
ITSN2	intersectin 2	18	26	8
JUP	junction plakoglobin	5	1	0
KANK2	KN motif and ankyrin repeat domains 2	3	4	2
KCNIP3	Kv channel interacting protein 3, calsenilin	0	1	1
KCNIP4	Kv channel interacting protein 4	0	1	0
MAPT	microtubule-associated protein tau	7	11	7
METL	Met1 methyltransferase	3	0	1
NCSTN	nicastrin	3	3	0
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8	1	1	0
NOS3	nitric oxide synthase 3 (endothelial cell)	1	1	2
NOTCH1	notch 1	6	5	2
OXCT1	3-oxoacid CoA transferase 1	2	3	0
PARL	presenilin associated, rhomboid-like	1	0	1
PDCD4	programmed cell death 4	1	2	1
PEN2	presenilin enhancer gamma secretase subunit	1	0	0
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	7	7	3
PKP4	plakophilin 4	6	7	6
PLD1	phospholipase D1	4	3	0
PRAM1	PML-RARA regulated adaptor molecule 1	9	30	4
PRDX1	peroxiredoxin 1 PAG	1	0	0
PRDX2	peroxiredoxin 2	0	2	0
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	2	2	2
PSEN1	presenilin 1	0	0	2
PSEN2	presenilin 2	1	0	0
PSMA5	proteasome (prosome, macropain) subunit, alpha type, 5	1	1	0
PSMB1	proteasome (prosome, macropain) subunit, beta type, 1	1	0	0
RAB11A	RAB11A	1	3	0
RAB3A	RAB3A, member RAS oncogene family	0	0	2
RAD23A	RAD23 homolog A (S. cerevisiae)	1	3	0

RHEB	Ras homolog enriched in brain	1	2	0
RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1	5	5	2
RMDN3	regulator of microtubule dynamics 3	0	5	2
RNF32	ring finger protein 32	1	4	4
RYR2	ryanodine receptor 2 (cardiac)	13	24	11
SCN1A	sodium channel, voltage-gated, type I, alpha subunit	7	17	9
SRI	Sorcin	0	0	0
ST13	suppression of tumorigenicity 13	2	7	1
STAMBPL1	STAM binding protein-like 1	1	3	0
STX1A	Syntaxin 1A	0	3	4
STX5	Syntaxin 5	2	6	0
TCF4	transcription factor 4	0	6	4
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	1	6	8
TDP2	tyrosyl-DNA phosphodiesterase 2	0	0	4
TGFBR1	transforming growth factor, beta receptor 1	1	3	0
TMED10	TMP21	1	3	0
TPTE2	TM phosphoinositide 3-phosphatase and tensin homolog 2	3	0	3
TRAF2	TNF receptor-associated factor 2	7	2	0
TRAF6	TNF receptor-associated factor 6	1	3	0
TUBA1B	tubulin, alpha 1b	2	1	1
UBC	ubiquitin C	5	0	0
UBQLN1	ubiquilin 1	0	2	0
UMPS	uridine monophosphate synthetase	1	2	0
YME1L1	YME1-like 1 ATPase	6	1	0

By comparing the Ubpred predicted sites of ubiquitination with published sites of ubiquitination the effectiveness of the Ubpred software can be analysed. The K377 residue of RIP1 was given a high confidence rating for ubiquitination and is a known target of K63-linked polyubiquitination [Ea *et al* 2006]. However, Ubpred gave a

low confidence rating for ubiquitination of TRAF6 at residue K124, which is the site of TRAF6 autoubiquitination [Lamothe *et al* 2007]. Meanwhile the site of TRAF2 K63-linked polyubiquitination at K31 [Li *et al* 2009] was not predicted to be a site of ubiquitination. Dystrobrevin was predicated to have 14 possible sites of ubiquitination (2 with low (L) confidence, 8 with medium (M) and 4 with a high (H) confidence) using Ubpred analysis but there are no published sites of dystrobrevin ubiquitination according to the BioGRID. Ubpred analysis of isoform A of APP predicts the APP contains 16 possible sites of ubiquitination (3L, 8M, 5H). This APP isoform has been shown to be ubiquitinated at residues K751 and K763 [Beltrao *et al* 2012]; both of these sites were given a medium confidence of ubiquitination. UMPS was predicted to have 3 potential sites of ubiquitination at residues K219, K223 and K314 by Ubpred and these sites of have been shown to undergo ubiquitination [Beltrao *et al* 2012]. However, another 18 sites have been published to undergo ubiquitination [Beltrao *et al* 2012; Stes *et al* 2014; Udeshi *et al* 2013; Kim *et al* 2013]. GCDH was predicted to have 3 lysine residues that are sites of ubiquitination (K32, K42 and K361). Two of these sites (K31 and K361), plus an additional 5 residues (K163, K202. K240, K371 and K377), are published site of GCDH ubiquitination [Udeshi *et al* 2013; Stes *et al* 2014]. CYP2C18 and CYP2C8 were both predicted to a solitary potential site of ubiquitination and there are no published sites of ubiquitination for either protein while ICAM5, which had no predicted sites of ubiquitination, is also not known to undergo ubiquitination. These data demonstrates that the complexity of the ubiquitination system within a cell cannot easily be replicated using *in silico* methods. The Ubpred system has some success in predicting sites of protein ubiquitination but Ubpred can both over- and under-estimate the likelihood of a protein being polyubiquitinated.

4.3 $P75^{NTR}$ Does Not Interact With PS1 Holoprotein or PS1 ΔCUE Mutant

The cleavage of some γ-secretase substrates is known to be regulated by K63-linked polyubiquitination including $P75^{NTR}$, IL-1R1 and TGFβR1 [Powell *et al* 2009; Twomey *et al* 2009; Gudey *et al* 2014]. Given that the PS1 CUE domain interacts with K63-linked polyubiquitin chains it was decided to investigate whether the CUE domain was required for the interaction between PS1 and interact with any of these γ-secretase substrates. To investigate this interaction HEK293T cells were transiently co-transfected with pRK5-$P75^{NTR}$ and pcDNA3.1-PS1 WT, pcDNA3.1-PS1 ΔCUE or pcDNA3.1-PS1 F283A/P284A/V309A/S310A **(Figure 4.1)**. PS1 has previously been shown to interact with the $P75^{NTR}$ after pre-treatment with the $P75^{NTR}$ ligand NGF [Powell *et al* 2009] so the cells were treated with NGF (100 ng/ml) for 20 mins prior to harvesting. Expression of $P75^{NTR}$ and the PS1 constructs was confirmed by western blotting and anti-β-actin was used as a loading control **(lower panels of Figure 4.1)** before an equivalent amount of each lysate was subject to immunoprecipitation with an anti-$P75^{NTR}$ antibody. After separation by SDS-PAGE the co-immunoprecipitation of PS1 with $P75^{NTR}$ was confirmed by western blotting with 614.1 anti-PS1 NTF and anti-PS1 CTF antibodies; equal immunoprecipitation of $P75^{NTR}$ was confirmed by immunoblotting with an anti-$P75^{NTR}$ antibody. Bands equivalent to both the PS1 NTF and PS1 CTF fragments were immunoprecipitated with $P75^{NTR}$ **(upper panels of Figure 4.1)**. However, no bands equivalent to the PS1 holoprotein or the PS1 ΔCUE mutant could be seen on the blot, which suggests that $P75^{NTR}$ does not interact with full length PS1 and as such deletion of the PS1 CUE domain has no effect on the interaction between $P75^{NTR}$ and PS1.

4.4 IL-1R1 Does Not Interact with PS1 Holoprotein nor PS1 ΔCUE Mutant

IL-1R1 is another γ-secretase substrate which is known to undergo K63-linked polyubiquitination [Twomey *et al* 2009]. While we have previously shown that the PS1 CUE domain is dispensable for the γ-secretase mediated cleavage of IL-1R1 **(Figure 3.15)** it was decided to investigate whether the PS1 CUE domain had any effect on regulating the interaction between PS1 and IL-1R1. HEK293T cells were transiently transfected with pcDNA3-IL-1R1 and either pcDNA3.1-PS1 WT, pcDNA3.1-PS1 ΔCUE or pcDNA3.1-PS1 F283A/P284A/V309A/S310A **(Figure 4.2)**. Cells were pre-treated with IL1 (10 ng/ml) for 15 mins prior to lysing. The cell lysates were subject to SDS-PAGE and expression of IL-1R1 and the PS1 constructs was demonstrated by western blotting with anti-IL-1R1 C20 and anti-PS1 CTF antibodies; anti-β-actin was used as a loading control **(lower panels Figure 4.2)**. After expression had been confirmed an equal amount of each cell lysate was subject to immunoprecipitation with anti-IL-1R1 C20 antibody, the immunipurified proteins were separated on a 12% gel by SDS-PAGE and co-immunoprecipitation of PS1 and IL-1R1 was demonstrated by western blotting with anti-PS1 CTF antibody; equal immunoprecipitation of IL-1R1 was shown by probing with anti-IL-1R1 antibody **(upper panels Figure 4.2)**. There are no bands equivalent to either the PS1 holoproteins, or to the PS1 ΔCUE mutant present **(Figure 4.2)**. These data suggest that IL1-R1 does not strongly interact with PS1 holoprotein and as such deletion of the PS1 CUE domain does not play a role in regulating the interaction between PS1 and IL1-R1. Therefore, it was concluded that the PS1 CUE domain is not required for regulating the interaction between PS1 and the K63-linked polyubiquitinated γ-secretase substrates $P75^{NTR}$ or IL-1R1. As the PS1 CUE domain has previously been

shown to be dispensable for the γ-secretase cleavage of APP, Notch and IL-1R1, these data reinforce the theory that the PS1 CUE domain is involved in γ-secretase independent PS1 function(s).

4.5 TRAF6 E3 Ligase activity is Required For interaction between TRAF6 and PS1

TRAF6 has been shown to interact with and ubiquitinate PS1, allowing it to regulate the cleavage of γ-secretases substrates such as TGFBR1, IL-1R1 and $P75^{NTR}$. [Elzinga *et al* 2009; Powell *et al* 2009; Gudey *et al* 2014]. TRAF6 is an E3 ligase that facilitates K63-linked polyubiquitination of its substrates, which requires residue C70; in addition to this TRAF6 undergoes auto-ubiquitination at its K124 residue [Lamothe *et al* 2007]. Since the CUE domain of PS1 selectively binds to K63-linked polyubiquitin chains it was next decided to investigate whether the PS1 CUE domain is responsible for the interaction between PS1 and TRAF6. To achieve this pRK5-FLAG-TRAF6, the E3 ligase defective mutant pRK5-FLAG-TRAF6 C70A and the auto-ubiquitination mutant pRK5-FLAG-TRAF6 K124R were co-expressed in HEK293T cells with either pcDNA3.1-PS1 WT or pcDNA3.1-PS1 ΔCUE. After overexpression was confirmed through western blotting **(lower panels of Figure 4.8)** the cell lysates were immunoprecipitated using anti-FLAG antibody and after SDS-PAGE the co-immunoprecipitation of PS1 was determined by immunoblotting with an anti-PS1 NTF antibody. Both PS1 **(lanes 3 and 5 of upper panel of Figure 4.3)** and PS1 ΔCUE **(lanes 5 and 8 of upper panel of Figure 4.3)** co-immunoprecipitated with TRAF6 and TRAF6 K124R. However, neither PS1 nor PS1 ΔCUE were co-immunoprecipitated with the TRAF6 C70A mutant **(lanes 4 and 7 of upper panel Figure 4.3)**. This result suggests that neither TRAF6 auto-ubiquitination nor PS1 CUE domain function are required or necessary for the

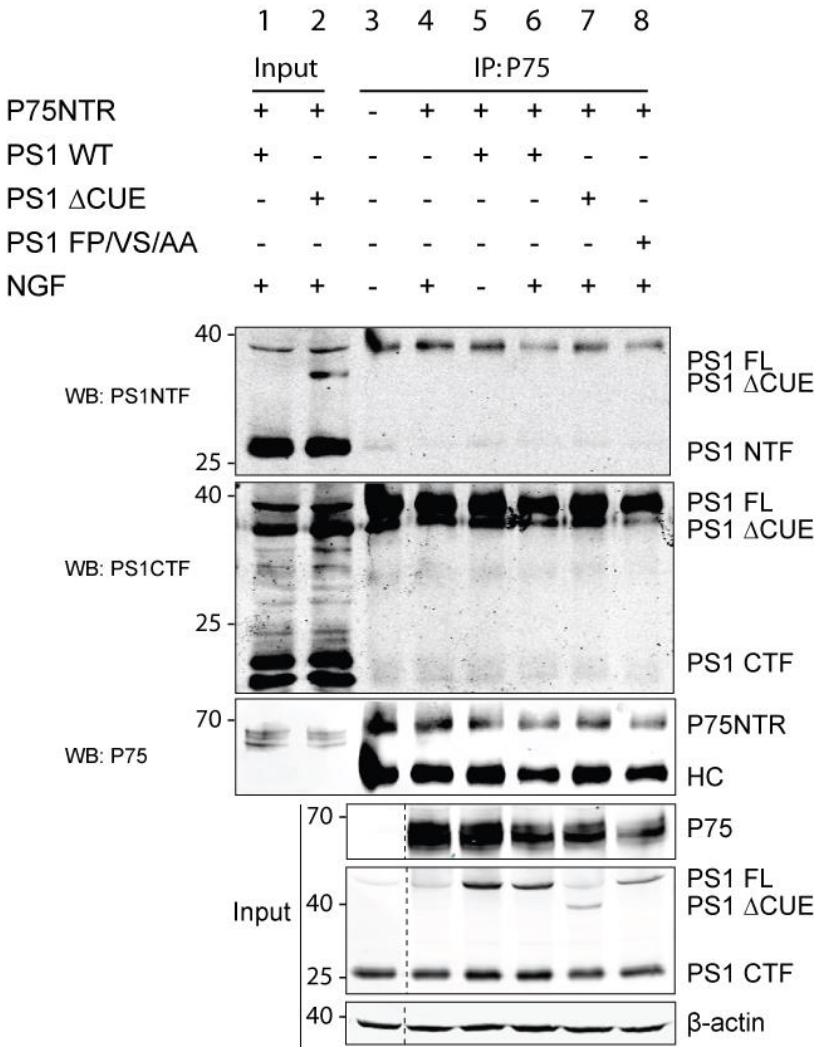


Figure 4.2 P75^{NTR} does not interact with PS1 nor do PS1 CUE domain mutants. HEK293T cells were transiently co-transfected with pRK5-P75^{NTR} and pcDNA3.1-PS1 WT, pcDNA3.1-PS1 ΔCUE or pcDNA3.1-PS1 F283A/P284A/V309A/S310A. 36 hours after transfection the cells were treated with 100 ng/ml NGF for 20 mins before the cells were harvested. The cells were lysed in lysis buffer and proteins were separated by SDS-PAGE in 12% gels. Overexpression was determined by immunoblotting with anti-P75^{NTR} and anti-PS1 CTF antibodies, anti-β-actin antibody was used to show equal loading (lower panels). Once overexpression had been shown, equivalent amounts of cell lysate were subjected to immunoprecipitation with anti-P75^{NTR} antibody, the immunopurified proteins were separated by SDS-PAGE on 12% gels. Co-immunoprecipitation was determined by western blotting with anti-PS1 NTF and anti-PS1 CTF antibodies; immunoprecipitation of P75^{NTR} was shown by western blotting with anti-P75^{NTR} antibody. FL: full length; HC: IgG heavy chain; CTF: C-terminal fragment; NTF: N-terminal fragment; IP: immunoprecipitation; NGF: nerve growth factor; NTR: neurotrophin receptor; WB: western blot; WT: wild type. Data are representative of experiments (n=2).

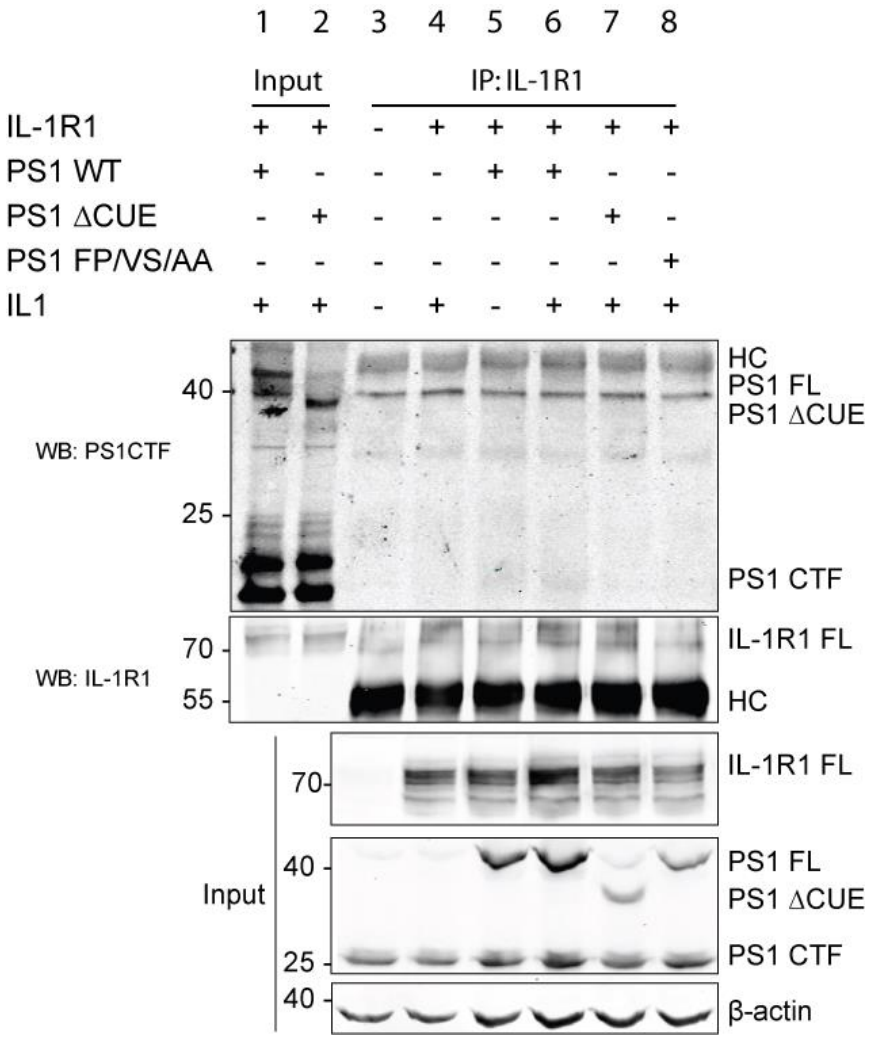


Figure 4.3 IL-1R1 does not interact with PS1 and PS1 CUE domain mutants. HEK293T cells were transiently co-transfected with pcDNA3-IL-1R1 and pcDNA3.1-PS1 WT, pcDNA3.1-PS1 ΔCUE or pcDNA3.1-PS1 F283A/P284A/V309A/S310A. 36 hours after transfection the cells were treated with 10 ng/ml IL1 for 15 mins before the cells were harvested. The cells were lysed in lysis buffer and proteins were separated by SDS-PAGE in 12% gels. Overexpression was determined by immunoblotting with anti-IL-1R1 C20 and anti-PS1 CTF antibodies, anti-β-actin antibody was used to show equal loading (lower panels). Once overexpression had been shown, equivalent amounts of cell lysate were subjected to immunoprecipitation with anti-IL-1R1 C20 antibody, the immunopurified proteins were separated by SDS-PAGE on 12% gels. Co-immunoprecipitation was determined by western blotting with anti-PS1 CTF antibody; immunoprecipitation of IL1-R1 was shown by western blotting with anti-IL-1R1 C20 antibody. FL: full length; HC: IgG heavy chain; CTF: C-terminal fragment; IP: immunoprecipitation; WB: western blot; WT: wild type. Data are from a representative experiment (n=3).

interaction between PS1 and TRAF6. It does suggest that TRAF6 E3 ligase activity is required for the interaction between TRAF6 and PS1.

4.6 Mutation of Conserved PS1 CUE Domain Motifs Affect PS1-TRAF6 Interaction

To further investigate the interaction between TRAF6 and PS1 it was decided to co-express TRAF6 with three PS1 CUE domain point mutants: PS1 F283A/P284A, PS1 V309A/S310A and PS1 F283A/P284A/V309A/S310A. In these mutants the main motifs in the PS1 CUE domain are either mutated single or together. As the TRAF6 C70A mutation has been shown to prevent the interaction between PS1 and TRAF6 this was used as a negative control for this co-immunoprecipitation experiment. HEK293T cells were transiently transfected with pRK5-FLAG-TRAF6 or pRK5-FLAG-TRAF6 C70A and co-transfected with either PS1 WT, PS1 F283A/P284A, PS1 V309A/S310A or PS1 F283A/P284A/V309A/S310A. After overexpression was confirmed via western blotting **(Lower panels of Figure 4.4)** the cell lysates were immunoprecipitated with an anti-FLAG antibody. PS1 and TRAF6 co-immunoprecipitated together and the TRAF6 C70A mutation again disrupted this interaction **(upper panel of Figure 4.4)**. Both PS1 F283A/P284A and PS1 V309A/S310A CUE domain mutants were co-immunoprecipitated with TRAF6 to a similar level as PS1 **(compare lanes 5 and 6 to lane 3 of upper panel of Figure 4.4)**, though there was variability between the different experiments. This result is unusual as removal of the entire CUE domain had no effect on the PS1-TRAF6 interaction **(Figure 4.3)** but mutation of the two CUE domain motifs caused a reduction in this interaction. This suggests that the interaction between PS1 and TRAF6 may be regulated by an intermediate protein or proteins.

4.7 TRAF6 Interacts With PS1 N-Terminal and C-Terminal Fragments

TRAF6 is known to interact with PS1 and the intracellular loop domain of PS1 is known to contain a TRAF6 interacting motif (PEERGV) at residues 374-379 [Powell *et al* 2009]. To further investigate the site of the interaction between PS1 and TRAF6, plasmids that express the PS1 NTF or the PS1 CTF domains were used (NSE-PS1 NTF and NSE-PS1 CTF, respectively). In addition, a PS1 mutant which had residues 305-372 from the intracellular loop domain of PS1 were removed (pCDNA3.1-PS1 ΔLoop) was used. This mutant removes the final 10 residues from the PS1 CUE domain including the essential V309/S310 motif of the CUE domain. In HEK293T cells pRK5-FLAG-TRAF6 was co-expressed with these PS1 mutants, pcDNA3.1-PS1 and pRK5-FLAG-TRAF6 C70A were used as positive and negative controls for the experiment. The NSE-PS1 NTF and NSE-PS1 CTF constructs did not overexpress as well as the pcDNA3.1-PS1 WT or pcDNA3.1-PS1 ΔLoop constructs **(Figure 4.5)**. These lysates were used in a co-immunoprecipitation assay using anti-FLAG antibody and after separation by SDS-PAGE the co-immunoprecipitation of TRAF6 and PS1 was detected by western blotting with both PS1 NTF and PS1 CTF antibodies. Both PS1 NTF and PS1 CTF weakly co-immunoprecipitated with TRAF6, however, these constructs were also weakly overexpressed **(input panels of Figure 4.5)**. This result suggests that TRAF6 can interact with both the PS1 NTF and CTF fragments at a similar level to one another The PS1 ΔLoop protein strongly co-immunoprecipitated with TRAF6 **(Figure 4.5, lane 5 upper panel)**. This result suggests that deletion of this part of the PS1 intracellular loop domain may cause an increased interaction between PS1 and TRAF6. This deleted region of the protein includes a part of the PS1 CUE domain and mutation of the two motifs within the PS1 CUE domain has been shown to cause

a reduction in the binding of PS1 to TRAF6 **(Figure 4.4)**. The deletion of part of the PS1 intracellular loop ends two residues before the TRAF6 interaction motif [Elzinga *et al* 2009; Powell *et al* 2009] which may explain the increased interaction between the PS1 ΔLoop mutant and TRAF6. This PS1 intracellular loop domain deletion may make the TRAF6 interaction motif more available for TRAF6 or deletion of the V309/S310 motif of the PS1 CUE domain may reduce the interaction of PS1 with another protein which regulates the interaction between PS1 and TRAF6.

4.8 Mutation of Conserved PS1 CUE Domain Motifs Has No Effect On TRAF6 Mediated Stability of PS1

Overexpression of TRAF6 with PS1 has previously been shown to help increase the stability of the PS1 holoprotein [Yan *et al* 2013] and deletion of the PS1 CUE domain has no effect on the TRAF6-mediated increase in stability of the PS1 holoprotein [Yan unpublished material]. However, since mutation of both PS1 CUE domain motifs caused a reduction in the interaction with PS1 and TRAF6 it was decided to investigate whether these mutations would have any effect on PS1 stability. HEK293T cells were transfected with pRK5-FLAG-TRAF6 and co-transfected with pcDNA3.1-PS1 or pcDNA3.1-PS1 F283A/P284A/V309A/S310A. Cell cultures were treated with the translation inhibitor cycloheximide (CHX) at differing time points. When expressed alone PS1 holoprotein was mostly turned over by 8 hours but when co-expressed with TRAF6 PS1 holoprotein was still present at high levels even after 24 hours of treatment with CHX. Similarly expression of the PS1 F283A/P284A/V309A/S310A was still present at 24 hours when co-expressed with TRAF6 suggesting that mutation of these residues had no effect on PS1 stability. This means that despite the reduction in the interaction between PS1 and

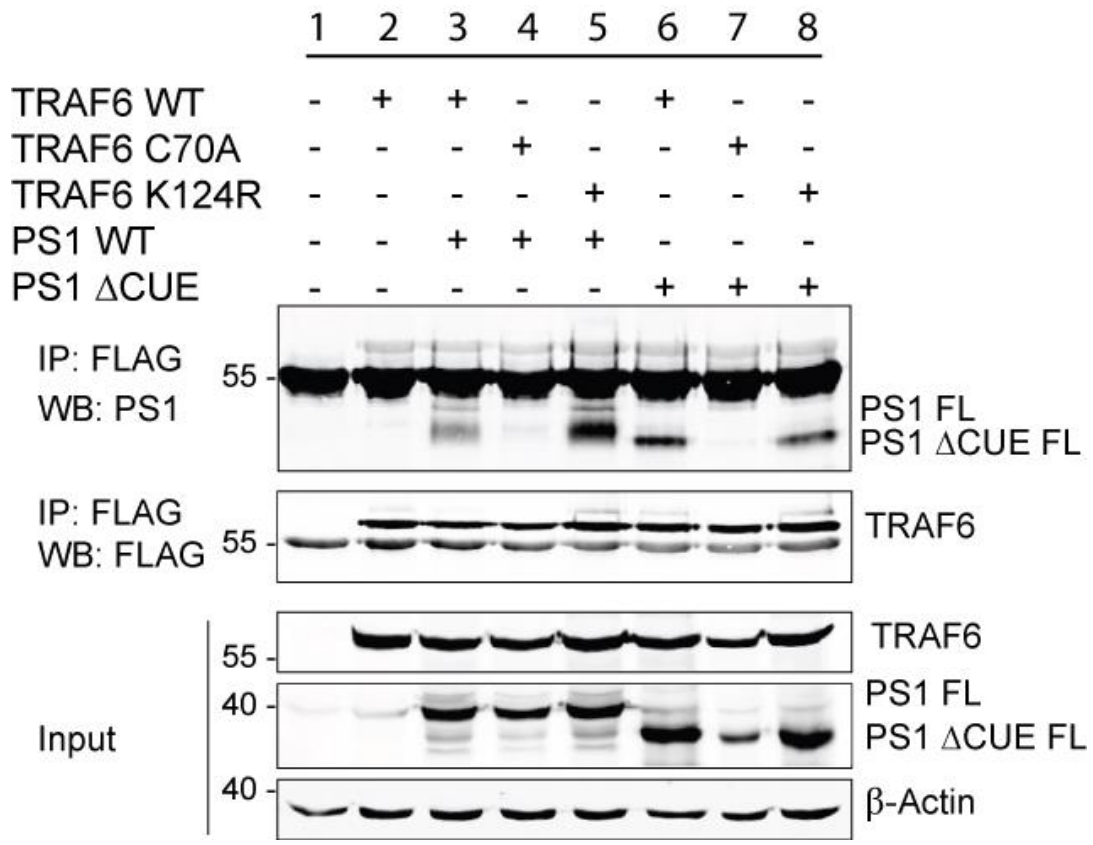


Figure 4.4 TRAF6 C70 residue is required for TRAF6-PS1 interaction. HEK293T cells were transiently co-transfected with pRK5-FLAG-TRAF6WT, pRK5-FLAG-TRAF6C70A or pRK5-FLAG-TRAF6K124R- and with either pcDNA3.1-PS1 WT or pcDNA3.1-PS1 ΔCUE. 36 hours after transfection the cells were lysed in Lysis buffer. The lysates were run on 12% SDS-PAGE gels and overexpression was detected with anti-FLAG or 614.1 anti-PS1 NTF antibodies, equal loading was confirmed by blotting with anti-β-actin antibody (input panels). After expression was confirmed an equivalent amount of protein (1000 μg) was subject to immunoprecipitation with anti-FLAG antibody. Immunoprecipitated proteins were separated on 12% SDS-PAGE gels and co-immunoprecipitation was detected by probing with 614.1 anti-PS1 NTF antibody. Immunoprecipitation of TRAF6 was detected by immunoblotting with anti-FLAG antibody. FL: full length; HC: IgG heavy chain; IP: immunoprecipitation; WB: western blot; WT: wild type. Data are from a representative experiment (n=3).

TRAF6 when these CUE domain residues are mutated that it has no effect on the TRAF6-mediated PS1 holoprotein stability.

4.9 TRAF2 Promotes K63-linked Polyubiquitination of Presenilin-1

Previous work in our lab has shown that PS1 interacts with TRAF2 and TRAF6 [unpublished data; Powell *et al* 2009]. Like TRAF6, TRAF2 is an E3 ligase that catalyses K63-linked polyubiquitin of its substrates. TRAF6 has previously been shown to promote the K63-linked polyubiquitination of PS1 [Yan *et al* 2013; Gudey *et al* 2014]. To investigate whether the interaction of TRAF2 with PS1 would also lead to the TRAF2 mediated ubiquitination of PS1, HEK293T cells expressing pcDNA3.1-PS1 WT were co-transfected with HA-ubiquitin or a K63R ubiquitin mutant, which cannot form K63-linked polyubiquitin chains, and with pRK5-FLAG-TRAF2 WT or a dominant negative mutant pRK5-FLAG-TRAF2DN, in which the RING domain and the Zinc Finger domains of TRAF2 (residues 1-289) have been deleted. The cells were lysed under denaturing conditions, the lysates were separated by SDS-PAGE and protein overexpression was confirmed by western blotting with anti-FLAG and anti-PS1 NTF antibody **(Figure 4.7 lower panels)**. Once overexpression was demonstrated the lysates were used in an ubiquitination assay. The equivalent amounts of cell lysate were immunoprecipitated with anti-PS1 NTF antibody and separation by SDS-PAGE. PS1 ubiquitination was detected by immunoblotting with an anti-HA antibody. Co-expression of pcDNA3.1-PS1 with pRK5-FLAG-TRAF2 and HA-Ub increased the formation of polyubiquitinated forms of PS1 **(Figure 4.7)**, however when co-expressed with either pRK5-TRAF2DN or HA-Ub K63R mutant there was a dramatic reduction in PS1 ubiquitination. These data show that like TRAF6, TRAF2 is able to promote K-63-linked polyubiquitination of PS1.

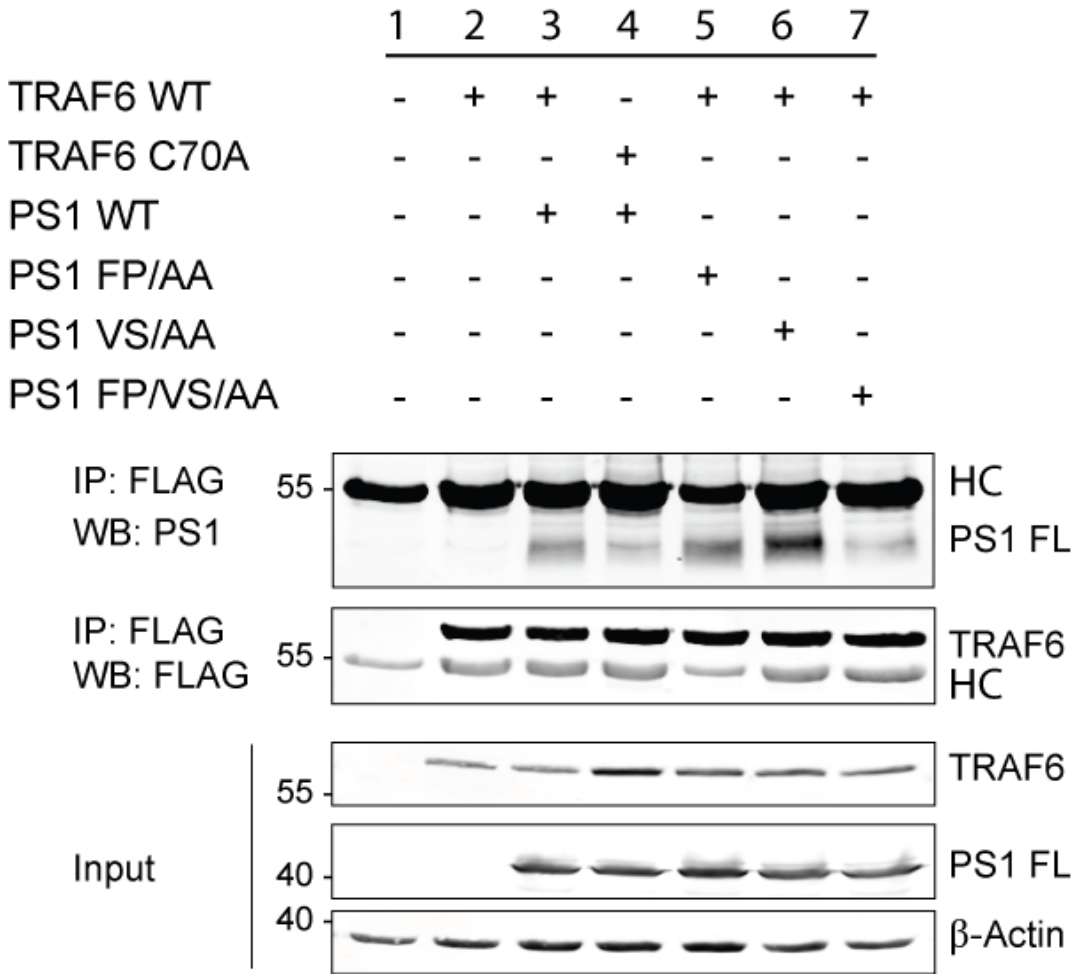


Figure 4.5 The PS1 F283A/P284A/V309A/S310A mutant shows reduced interaction with TRAF6. HEK293T cells were transiently co-transfected with either pRK5-FLAG-TRAF6WT or pRK5-FLAG-TRAF6C70A and with pcDNA3.1-PS1 WT, pcDNA3.1-PS1 F283A/P284A, pcDNA3.1-PS1 V309A/S310A or pcDNA3.1-PS1 F283A/P284A/V309A/S310A. 36 hours after transfection the cells were lysed in Lysis buffer. The lysates were run on 12% SDS-PAGE gels and overexpression was detected with anti-FLAG or 614.1 anti-PS1 NTF antibodies, equal loading was confirmed by blotting with anti-β-actin antibody (input panels). After expression was confirmed an equivalent amount of protein (1000 μg) was subject to immunoprecipitation with anti-FLAG antibody. Immunoprecipitated proteins were separated on 12% SDS-PAGE gels and co-immunoprecipitation was detected by probing with 614.1 anti-PS1 NTF antibody. Immunoprecipitation of TRAF6 was detected by immunoblotting with anti-FLAG antibody. FL: full length; HC: IgG heavy chain; IP: immunoprecipitation; WB: western blot; WT: wild type. Data are from a representative experiment (n=3).

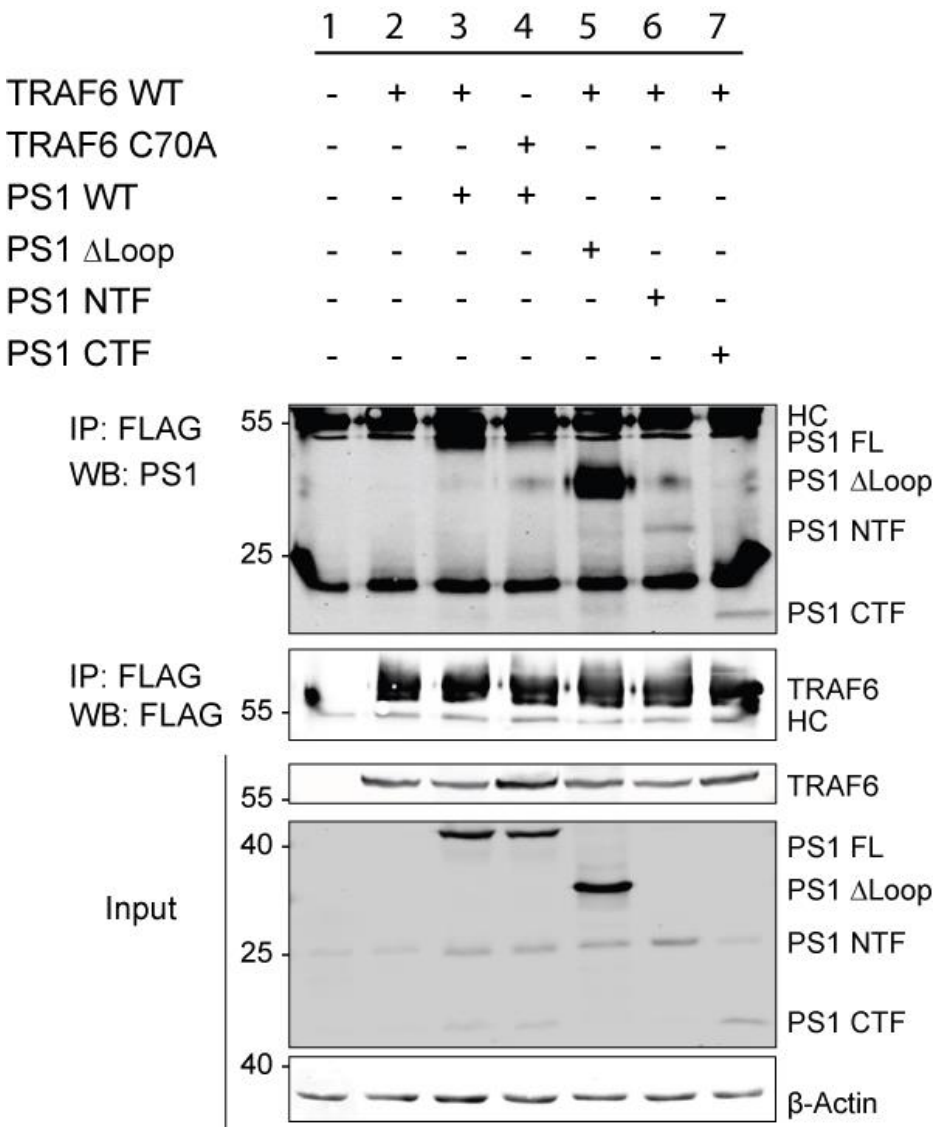


Figure 4.6 PS1 N-terminal and C-terminal fragments interact with TRAF6; PS1 ΔLoop mutant interacts with TRAF6. HEK293T cells were transiently co-transfected with either pRK5-FLAG-TRAF6WT or pRK5-FLAG-TRAF6C70A and with pcDNA3.1-PS1 WT, pcDNA3.1-PS1 ΔLoop, NSE-PS1 NTF or NSE-PS1 CTF. 36 hours after transfection the cells were lysed in Lysis buffer. The lysates were run on 12% SDS-PAGE gels and overexpression was detected with anti-FLAG, anti-PS1 CTF or 614.1 anti-PS1 NTF antibodies, equal loading was confirmed by blotting with anti-β-actin antibody (input panels). After expression was confirmed an equivalent amount of protein (1000 μg) was subject to immunoprecipitation with anti-FLAG antibody. Immunoprecipitated proteins were separated on 12% SDS-PAGE gels and co-immunoprecipitation was detected by probing with anti-PS1 CTF and 614.1 anti-PS1 NTF antibodies. Immunoprecipitation of TRAF6 was detected by immunoblotting with anti-FLAG antibody. FL: full length; HC: IgG heavy chain; IP: immunoprecipitation; NTF: N-terminal fragment; CTF: C-terminal fragment; WB: western blot; WT: wild type. Data are from a representative experiment (n=3).

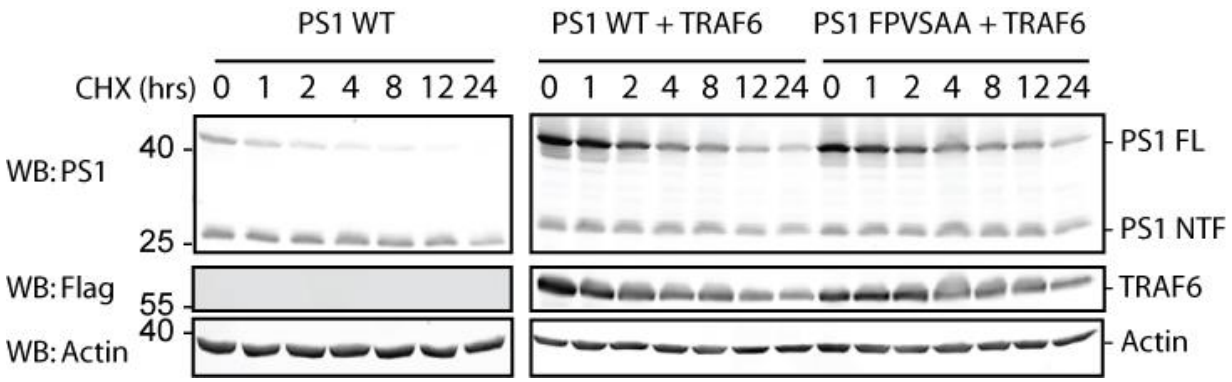


Figure 4.7 Mutation of conserved PS1 CUE domain motifs does not affect the TRAF6 mediated increase in PS1 FL stability. HEK293T cells were transiently transfected with pcDNA3.1-PS1 WT or co-transfected with pcDNA3.1-PS1 WT or pcDNA3.1-PS1 F283A/P284A/V309A/S310A and pRK5-FLAG-TRAF6WT. Cells were treated with 20 μg/ml cycloheximide (CHX) for the indicated time period. Cells were harvested 36 hours post-transfection, separated on 12% SDS-PAGE gels and immunoblotted with anti-FLAG or 614.1 anti-PS1 NTF antibodies. Equal loading was detected by probing with anti-β-actin antibody. CHX: cycloheximide; FL: full length; hrs: hours; NTF: N-terminal fragment; WB: western blot; WT: wild type. Data are from a representative experiment (n=3).

4.10 Deletion of the PS-1 CUE Domain Does Not Affect binding to TRAF2

As well as acting as an E3 ligase that can add K63-linked polyubiquitin chains to substrates, TRAF2 itself undergoes ubiquitination in both a K48- and K63-linked manner [Li et al 2009]. As TRAF2 has been shown to interact with PS1 [Powell unpublished material], it was decided to investigate whether this interaction requires the CUE domain of PS1. In HEK293T cells either pRK5-FLAG-TRAF2 or pRK5-FLAG-TRAF2DN were co-expressed with pcDNA3.1-PS1, pcDNA3.1-PS1 ΔCUE or pcDNA3.1-PS1 F283A/P284A/V309A/S310A **(Figure 4.8)**. The cells were lysed and lysates were separated by SDS-PAGE and overexpression was confirmed by western blotting with anti-FLAG and anti-PS1 CTF antibodies. After protein expression was confirmed the lysates were subject to a co-immunoprecipitation experiment using an anti-PS1 NTF antibody. The samples were separated via SDS-PAGE and the co-immunoprecipitation of TRAF2 with PS1 was confirmed by immunoblotting with an anti-TRAF2 antibody **(lower panels of Figure 4.8)**. In addition to this TRAF2 WT was immunoprecipitated with both the PS1 ΔCUE and PS1 F283A/P284A/V309A/S310A CUE domain mutants. Similarly to TRAF2DN, there does appear to be less TRAF2WT co-immunoprecipitated with the PS1 F283A/P284A/V309A/S310A mutant but also less PS1 F283A/P284A/V309A/S310A protein was immunoprecipitated. This result shows that the interaction of PS1 with TRAF2 is not controlled by the activity of the PS1 CUE domain.

4.11 Presenilin-1 CUE Domain Not Required For RIP1 Interaction

RIP1 is an adaptor protein that is involved in the transmission of tumour necrosis factor (TNF) signalling from TNF Receptors (TNFR) but is also involved in other signalling pathways [Humphries *et al* 2015]. RIP1 is known to undergo both K48-

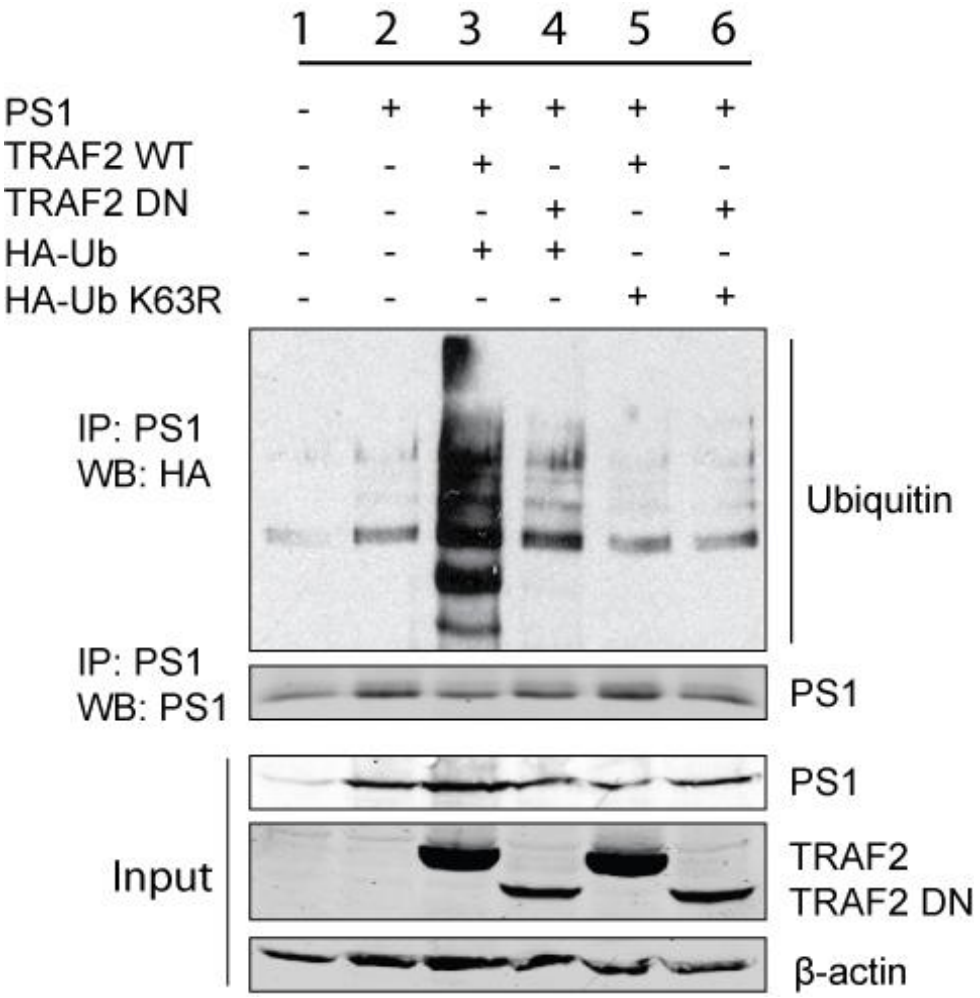


Figure 4.8 TRAF2 promotes K63-linked polyubiquitination of PS1. HEK293T cells were transiently co-transfected with pcDNA3.1-PS1 WT, either pRK5-FLAG-TRAF2 or pRK5-FLAG-TRAF2DN and with HA-Ub or HA-Ub K63R. 36 hours post-transfection the cells were lysed under strict denaturing conditions, equivalent amounts of lysate were separated on 12% SDS-PAGE gels and overexpression was detected with anti-FLAG and 614.1 anti-PS1NTF antibodies; equal loading was demonstrated with anti-β-actin antibody (input panels). After overexpression had been demonstrated an equivalent amount of each lysate (1000 μg) was subjected to immunoprecipitation with 614.1 anti-PS1 antibody. PS1 ubiquitination was detected with immunoblotting with anti-HA antibody, PS1 immunoprecipitation was detected with probing with 614.1 anti-PS1 NTF antibody. DN: dominant negative; IP: immunoprecipitation; WB: western blot; Ub: ubiquitin. Data are from a representative experiment (n=2).

and K63-linked polyubiquitination, and K63-linked ubiquitination of RIP1 at residue K377 has been shown to be essential for RIP1 function in signal transduction [Ea *et al* 2006]. Previous work in our lab has shown that RIP1 interacts with PS1 [unpublished data] and as RIP1 is known to undergo K63-linked polyubiquitination it was decided to investigate whether this ubiquitination event was required for the PS1-RIP1 interaction. HEK293T cells were transfected with either pRK5-RIP1 or the ubiquitination mutant pRK5-RIP1 K377R and co-transfected with pcDNA3.1-PS1, pcDNA3.1-PS1 ΔCUE or pcDNA3.1-PS1 F283A/P284A/V309A/S310A **(Figure 4.9)**. Overexpression of the various constructs was confirmed via western blotting and then the lysates were immunoprecipitated using anti-PS1 NTF antibody. After separation by SDS-PAGE the co-immunoprecipitation of RIP1 with PS1 was determined by immunoblotting for RIP1. Wild type RIP1 interacts with PS1 WT, PS1 ΔCUE and PS1 F283A/P284A/V309A/S310A **(Figure 4.9)**. In addition to this the RIP1 K377R mutant, which does not undergo K63 linked polyubiquitination, was also shown to interact with PS1 WT. These data shows that the interaction of RIP1 and PS1 is not mediated by the ubiquitination state of RIP1 or the functionality of the PS1 CUE domain.

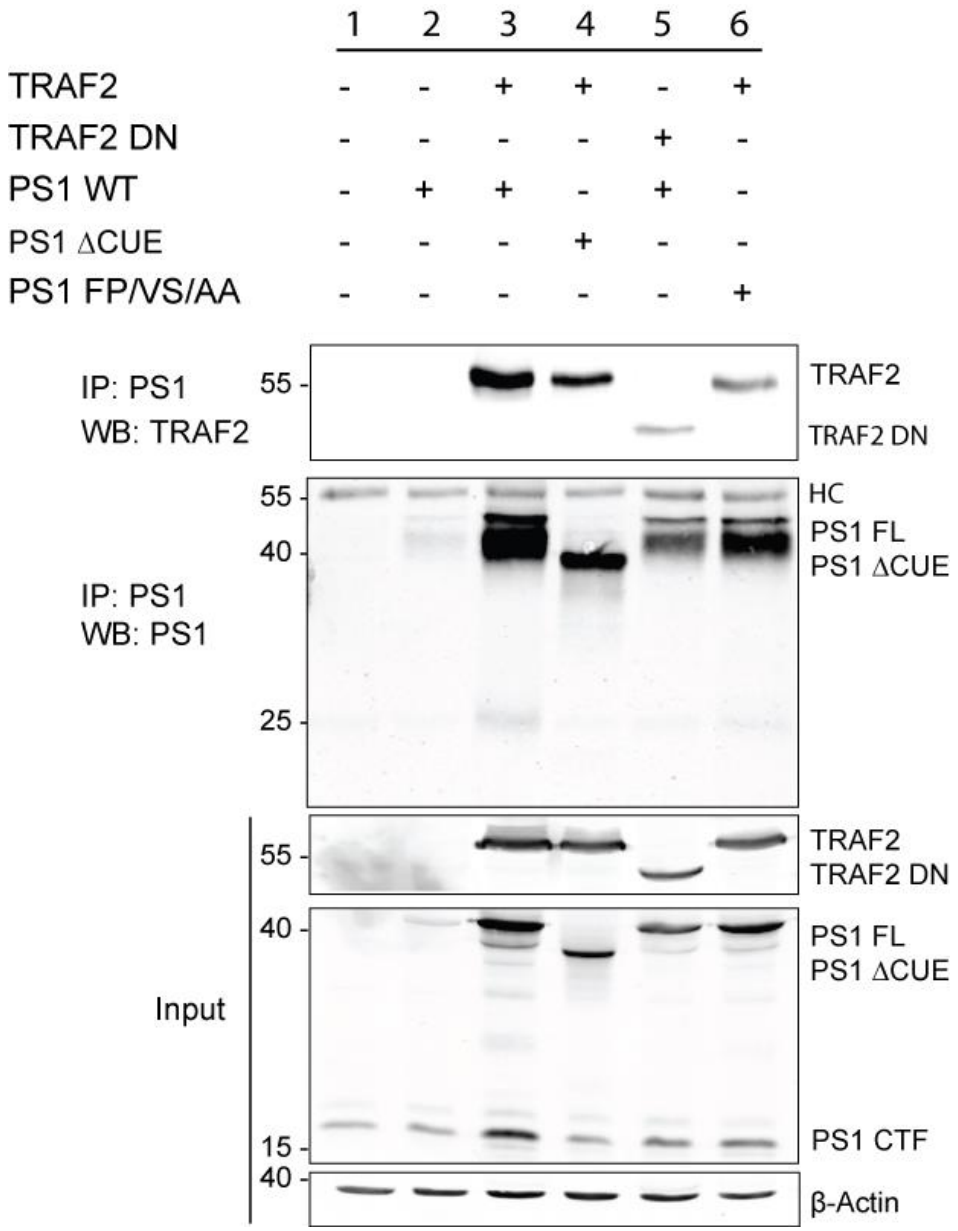


Figure 4.9 PS1 CUE domain is not required for PS1-TRAF2 interaction. HEK293T cells were transiently co-transfected with either pRK5-FLAG-TRAF2WT or pRK5-FLAG-TRAF2DN and with pcDNA3.1-PS1 WT, pcDNA3.1-PS1 ΔCUE or pcDNA3.1-PS1 F283A/P284A/V309A/S310A. 36 hours after transfection the cells were lysed in Lysis buffer. The lysates were run on 12% SDS-PAGE gels and overexpression was detected with anti-FLAG or anti-PS1 CTF antibodies, equal loading was confirmed by blotting with anti-β-actin antibody (input panels). After expression was confirmed an equivalent amount of protein (1000 μg) was subject to immunoprecipitation with anti-PS1 NTF antibody. Immunoprecipitated proteins were separated on 12% SDS-PAGE gels and co-immunoprecipitation was detected by probing with anti-TRAF2 antibody. Immunoprecipitation of PS1 was detected by immunoblotting with anti-PS1 CTF antibody. DN: dominant negative; FL: full length; HC: IgG heavy chain; IP: immunoprecipitation; WB: western blot; WT: wild type. Data are from a representative experiment (n=2).

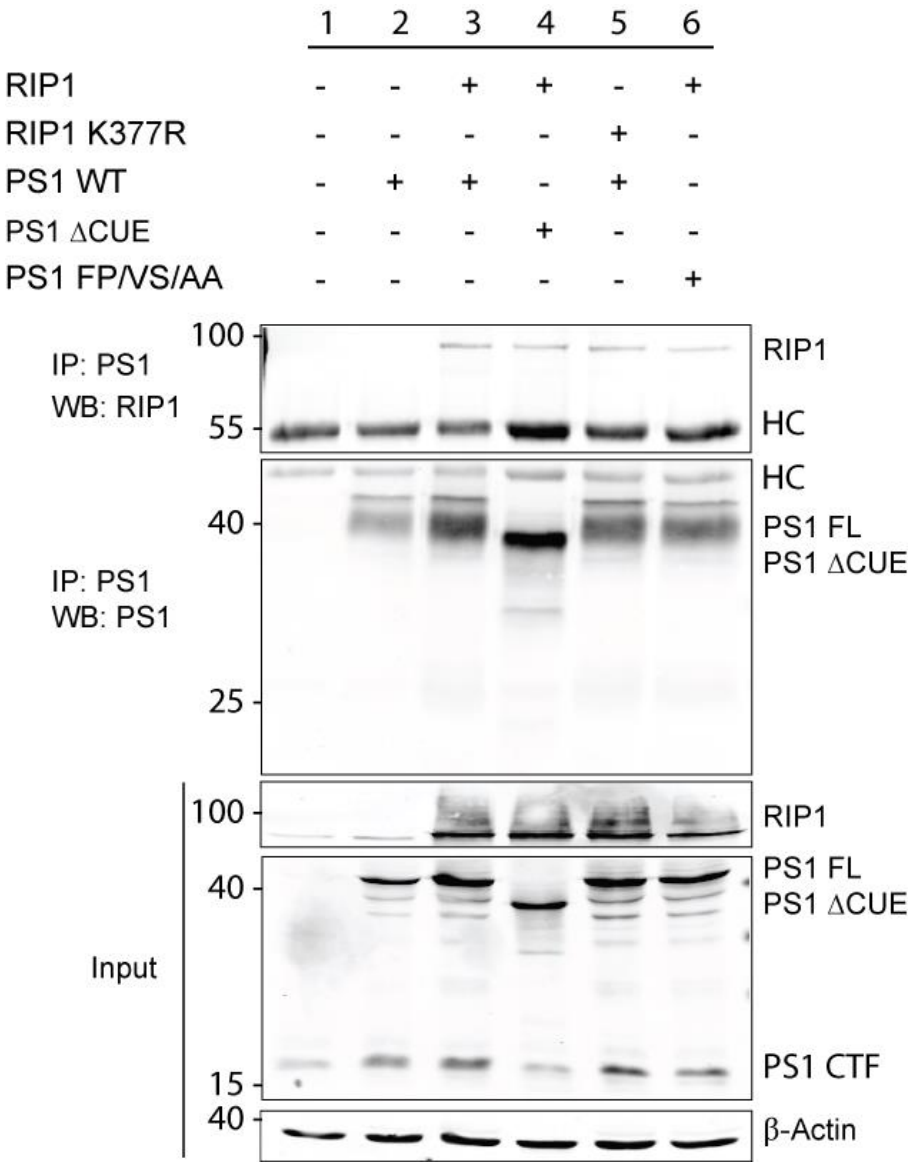


Figure 4.10 PS1 CUE domain is not required for PS1-RIP1 interaction. HEK293T cells were transiently co-transfected with either pRK5-RIP1 or pRK5-RIP1 K377R and with pcDNA3.1-PS1 WT, pcDNA3.1-PS1 ΔCUE or pcDNA3.1-PS1 F283A/P284A/V309A/S310A. 36 hours after transfection the cells were lysed in Lysis buffer. The lysates were run on 12% SDS-PAGE gels and overexpression was detected with anti-RIP1 or anti-PS1 CTF antibodies, equal loading was confirmed by blotting with anti-β-actin antibody (input panels). After expression was confirmed an equivalent amount of protein (1000 µg) was subject to immunoprecipitation with anti-PS1 NTF antibody. Immunoprecipitated proteins were separated on 12% SDS-PAGE gels and co-immunoprecipitation was detected by probing with anti-RIP1 antibody. Immunoprecipitation of PS1 was detected by immunoblotting with anti-PS1 CTF antibody. FL: full length; HC: IgG heavy chain; IP: immunoprecipitation; WB: western blot; WT: wild type. Data are from a representative experiment (n=2).

Discussion

Having shown that the presenilins contain K63-linked polyubiquitin chain binding CUE domains, in this chapter we began to investigate the function of the PS1 CUE domain. In the previous chapter it was shown that PS1 CUE domain function was not involved in PS1 endoproteolysis **(Figure 3.11)** or the γ-secretase cleavage of APP, IL-1R1 and Notch. In this chapter a list of known PS1 interacting proteins was compiled from previously published reviews [McCarthy *et al* 2009] and from the online database BioGRID (www.thebiogrid.org), which resulted in a list of 110 different proteins. The BioGRID, which also contains details of published posttranslational modifications, was then used to determine which of these PS1 interacting proteins are substrates of K63-linked polyubiquitination **(Table 4.1)**. Of these 110 PS1 interacting proteins 25 are not known to be ubiquitinated and 58 proteins were found to be ubiquitinated through high throughput mass spectrometric analysis. This analysis demonstrates that a lysine residue is ubiquitinated but does not show the type of ubiquitination that occurs there. Of the remaining proteins from this list 10 proteins were found to undergo K63-linked polyubiquitination **(Table 4.2)** and were considered to be potential PS1 CUE domain interacting proteins. Additionally, an ubiquitination site prediction tool (www.ubpred.org) was used to determine the number of potential sites of ubiquitination amongst these PS1 interacting proteins **(Table 4.3)**. Of the 110 proteins examined only 10 proteins were predicted to have no likely sites of ubiquitination.

Next to investigate the function of the PS1 CUE domain we used co-immunoprecipitation assays to determine whether deletion of the PS1 CUE domain or mutation of conserved PS1 CUE domain residues had any effect on regulating PS1 protein-protein interactions. There are 10 known PS1 interacting proteins that

are known to be K63-linked polyubiquitinated **(Table 4.2)**. From this list of 10 proteins we investigated whether the PS1 CUE domain is involved in regulating the interaction of PS1 with TRAF6, TRAF2 and RIP1. TRAF6 has previously been shown to interact with PS1 [Elzinga *et al* 2009; Powell *et al* 2009] and to promote the K63-linked polyubiquitination of PS1 [Yan *et al* 2013; Gudey *et al* 2014]. This interaction has been shown to be involved in regulating the γ-secretase cleavage of IL-1R1, $P75^{NTR}$ and TGFβR1 [Powell *et al* 2009; Powell *et al* 2009; Gudey *et al* 2014]. Co-expression of PS1 and TRAF6 has been shown to promote the K63-linked polyubiquitination of PS1 and also to increase the stability of the PS1 holoprotein [Yan *et al* 2013]. In this chapter we demonstrated that deletion of the PS1 CUE domain nor mutation of residue K124 of TRAF6, the site of K63-linked TRAF6 autoubiquitination, did not affect the interaction between PS1 and TRAF6 **(Figure 4.8)**. However, mutation of the C70 residue of TRAF6, which is essential for TRAF6 E3 function, caused a reduction in the binding of TRAF6 to both PS1 WT and the PS1 ΔCUE mutant **(Figure 4.8)**. Additionally we showed that single mutation of the F283/P284 or the V309/S310 CUE domain motifs did not cause a reduction in the interaction between PS1 and TRAF6 but mutation of both motifs in tandem (the PS1 F283A/P284A/V309A/S310A mutant) caused a reduction in this interaction **(Figure 4.9)**. Both the PS1 NTF and CTF fragments could be co-immunoprecipitated with TRAF6 **(lanes 6 and 7 of Figure 4.10)**. Deletion of residues 305-372 from the intracellular loop of PS1 caused an increased affinity of PS1 for TRAF6 **(lane 5 of Figure 4.10).** This partial deletion of the PS1 intracellular loop domain ends only 2 residues before the PS1 TRAF6 interacting motif (residues 374-379), which may explain the increased affinity of this PS1 mutant for TRAF6.

Mutation of the FANCD2 CUE domain leads to an increase in FANCD2 proteasomal degradation [Rego *et al* 2012]. Co-expression of TRAF6 with PS1 has been shown to promote the stability of the PS1 holoprotein [Yan *et al* 2013] and deletion of the PS1 CUE domain has previously been shown to have no effect on this process [Yan unpublished material]. However, since we have demonstrated here that the PS1 F283A/P284A/V309A/S310A CUE domain mutant shows a reduced interaction with TRAF6 it was decided to investigate whether TRAF6 could still promote an increase in holoprotein stability for this PS1 mutant. TRAF6 overexpression promoted an increased stability for both the PS1 WT and the PS1 F283A/P284A/V309A/S310A holoproteins **(Figure 4.13)**, suggesting that the decreased interaction between TRAF6 and the PS1 F283A/P284A/V309A/S310A mutant had no effect on this TRAF6 function.

The interaction between RIP1 and PS1 was not affected by PS1 CUE domain deletion or by mutation of the conserved PS1 CUE domain motifs **(Figure 4.3)**. Similarly, mutation of residue K377 within RIP1, a previously published site of RIP1 K63-linked polyubiquitination [Ea *et al* 2006], had no effect on the interaction between RIP1 and PS1. These data suggest that the interaction between PS1 and RIP1 is not determined by the ubiquitination state of RIP1. PS1 has previously been shown to be a substrate for TRAF6 mediated ubiquitination [Yan *et al* 2013; Gudey *et al* 2014]. Therefore, we next showed that PS1 is a substrate for TRAF2 mediated K63-linked polyubiquitination **(Figure 4.6)**. Next we showed that the PS1 CUE domain is dispensable for the interaction between PS1 and TRAF2 **(Figure 4.7)**. TRAF2 co-immunoprecipitated with PS1 WT and the PS1 ΔCUE and PS1 F283A/P284A/V309A/S310A CUE domain mutants and the TRAF2DN mutant (which is missing the RING and Zinc finger domains at the N-terminus of TRAF2)

was co-immunopurified with PS1 WT. These data suggest that the PS1 CUE domain is not involved in the interactions of PS1 with RIP1 or TRAF2.

Tollip negatively regulates β-catenin/Wnt signalling but deletion of the Tollip CUE domain abrogates this inhibition [Torún *et al* 2015]. Deletion of the TAB2 CUE domain causes a reduction in NFκB activity [Kishida *et al* 2005]. In CUEDC2 deletion of the CUE domain does not affect the binding of CUEDC2 with Cdc20 but instead this interaction is mediated by the Cdk1 phosphorylation of CUEDC2 [Gao *et al* 2011]. However, in this study both CUEDC2 and the presence of the CUEDC2 CUE domain were still required for dissociation of Mad2 from Cdc20. The CUE domain of CUEDC2 is required for the interaction of CUEDC2 and the progesterone receptor (PR) [Zhang *et al* 2007]. However, the CUE domain is not sufficient for the CUEDC2 mediated inhibition of progesterone receptor signalling [Zhang *et al* 2007]. These data suggest that the CUEDC2 CUE domain may affect function as a mediator of CUEDC2's protein-protein interactions. Deletion of the entire PS1 CUE domain did not affect the interaction of PS1 and TRAF6 **(Figure 4.8)** but mutation of the two conserved CUE domain motifs in tandem caused a reduction in this interaction **(Figure 4.9)**. From this unusual result we hypothesised that a PS1 CUE domain interacting protein may act as an intermediate in the interaction between PS1 and TRAF6. From these data the identity of PS1 CUE domain interacting protein or proteins remains unknown. Some unknown PS1 interacting protein may regulate the interaction between PS1 and TRAF6. This interaction could be unaffected by deletion of the PS1 CUE domain but the presence of a non-functional CUE domain may allow the interaction of other unidentified protein(s) with PS1 which negatively affect the PS1-TRAF6 interaction. This result suggests that the interaction between PS1 and TRAF6 may be mediated by a more complex system than a simple protein-

protein interaction. The Tollip CUE domain plays a role in modulating Tollip's interaction with the cell membrane as the competitive binding of ubiquitin to the Tollip CUE domain prevents the Tollip C2 domain from interacting with phosphoinositides [Mitra *et al* 2013]. Human isoT protein contains one inactive UBA domain (UBA1) and one active ubiquitin binding domain (UBA2) [Raasi *et al* 2005]. However, an isoT mutant which replaces the UBA2 domain with an additional UBA1 domain retains ubiquitin binding ability suggesting that isoT UBA function is dependent on non-UBA regions of the protein. These data demonstrate how the function of ubiquitin binding domains can be modulated by intra-protein or protein-protein interactions.

Of the proteins examined here only TRAF6 showed any change in its interaction with PS1 WT or with PS1 CUE domain mutants. However, the proteins studied here were PS1 interacting proteins that had been identified within our lab. Ten different PS1 interacting proteins were identified as undergoing K63-linked polyubiquitination **(Table 4.2)** and of these proteins a few are known to be involved in both γ-secretase –dependent and –independent functions of PS1. The interaction of PS1 and β-catenin is involved in γ-secretase independent PS1 functions [Raurell *et al* 2008] and should represent a target for future investigation of PS1 CUE domain function. BACE1 is a protease that is involved in ectodomain shedding in regulated intramembrane proteolysis [Kang *et al* 2008] and is thought of as the rate-limiting step in APP cleavage [Cole and Vassar 2007]. Fe65 is known to interact with the γ-secretase APP cleavage product AICD to regulate Neprilysin gene regulation [Vazquez *et al* 2009]. Tau is a protein that is found in the neurofibrillary tangles that are present in the brains of AD patients which also undergoes K63-linked polyubiquitination [Babu *et al* 2005]. The UBA domain containing protein p62 is

known to be required for correct turnover of K63-polyubiquitinated Tau [Babu *et al* 2008]. As these 3 proteins are involved in regulated intramembrane proteolysis, APP signalling and AD disease they represent strong targets for future investigation of PS1 CUE domain function.

The data shown here did not identify any PS1 CUE domain interacting proteins. To determine the identity of potential PS1 CUE domain interacting proteins it was decided to next use a high throughput proteomic approach. In the next chapter we will present proteomics data attained from mass spectrometry that we will use to investigate whether the PS1 CUE domain is involved in regulating intermolecular or intramolecular interactions of PS1.

5 Mass Spectrometry Analysis of PS1 Interacting Proteins

Introduction

Having first shown that the PS1 CUE domain selectively binds to K63-linked polyubiquitin chains, the PS1 CUE domain was shown to be dispensable for the interaction of PS1 with a number of PS1 interacting proteins which are known to undergo K63-linked polyubiquitination. While the interaction between PS1 and TRAF6 has been shown to be somewhat regulated by the PS1 CUE domain, this approach has not helped identify PS1-binding partners nor demonstrate the function of the PS1 CUE domain. To identify PS1 CUE domain binding proteins and to elucidate PS1 CUE domain function(s) it was decided to utilise a mass spectrometry-based proteomics approach. The purpose of this experiment was to isolate PS1 CUE domain interacting proteins from whole cell lysate using a recombinant bait protein which would include the PS1 CUE domain. After isolation of these PS1 CUE domain interacting proteins, mass spectrometry would be used to identify these interacting proteins. A further aim of the experiment was to determine whether the PS1 CUE domain is involved in the intermolecular or intramolecular interactions of PS1.

Mass spectrometry-based proteomic approaches typically involve the use of a bait protein that is either singly- [Shi *et al* 2011] or doubly-tagged [Tagwerker *et al* 2006]. Tandem affinity-purification (TAP) tags have often been used in the identification of interacting proteins [Tagwerker *et al* 2006; Guerrero *et al* 2006; Golebiowski *et al* 2010]. In these approaches the bait protein contains two different protein tags that are separated by a protease cleavage site. The bait protein and any bound proteins are first purified using one tag before this tag is removed by

treatment with the appropriate protease. The remainder of the bait protein can then be re-purified using the second epitope tag [Rigaut *et al* 1999]. The benefit of this sequential purification method is that it allows for a reduction in non-specific binding of proteins as it uses two different purification systems in tandem. There is a variation of this method has been used to aid in discovering ubiquitinated proteins where both the target protein(s) and ubiquitin are tagged with differing epitope tags [Maine *et al* 2010]. This approach allows for a protein sample to be enriched with ubiquitinated proteins and then ubiquitinated forms of the protein(s) of interest can be purified. This also allows for better identification of sites of ubiquitination within a protein of interest. Identification of general sites of ubiquitination within the proteome of cells have also been investigated through a tag-based mass spectrometry approach. In this approach GST-tagged repeats of different UBA domains have been incubated with whole cell lysate to enrich for ubiquitinated proteins prior to mass spectrometry analysis [Shi *et al* 2011]. Since a number of GST-tagged recombinant PS1 Loop containing proteins had already been created in our lab, it was decided to use this approach to attempt to identify PS1 CUE interacting proteins.

In this chapter an endogenous GST pulldown assay was validated, which uses the previously described recombinant GST-PS1-Loop and GST-PS1-Loop FP/VS/AA CUE domain mutant proteins. The GST-tagged proteins were first shown to bind to unknown proteins using Coomassie staining and the assay was validated by showing an interaction between recombinant GST-PS1-Loop and Rab11. Further validation of the methodology ruled out the need to use the DUB inhibitor, phenanthroline when lysing the HEK293T cells and the use of a glutathione elution buffer when removing bound proteins from the purification column. Three different samples were sent for mass spectrometry analysis: the negative control GST, the positive control GST-

PS1-Loop and the test sample CUE domain mutant GST-PS1-Loop FP/VS/AA. Once the mass spectrometry results were returned and analysed, it was concluded that the PS1 loop and CUE domains are not involved in regulating PS1-PS1 intramolecular interactions. The lists of proteins identified by mass spectrometry were analysed and broken down to show the proteins that interact with either the GST-PS1-Loop or the GST-PS1-Loop FP/VS/AA recombinant proteins. The molecular and biological functions of the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA interacting proteins were then investigated using a number of bioinformatics approaches to help elucidate the function of the PS1 CUE domain.

5.1 Validation of GST-Pulldown Assay

To determine the identity of PS1 CUE domain interacting proteins a GST-pulldown assay utilizing endogenous human proteins was first developed. This assay involved the use of the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA recombinant proteins that have been previously described in Section 3.7. These recombinant proteins contain the entire intracellular PS1 loop domain (residues 265-380) including the PS1 CUE domain; the GST-PS1-Loop-FP/VS/AA protein contains mutations at conserved CUE domain residues. For these experiments recombinant GST, GST-PS1-Loop and GST-PS1-Loop FP/VS/AA were purified as previously described in Sections 3.8 and 3.9. The elutions of both GST-PS1-Loop and GST-PS1-Loop FP/VS/AA proteins were separated by SDS-PAGE on a 12% gel and stained with a coomassie stain to compare the amount of recombinant protein present in each elution; the elutions of both GST-PS1-Loop and GST-PS1-Loop FP/VS/AA were comparable in protein concentration **(Figure 5.1A)**. Next, the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA recombinant proteins were used in the GST-pulldown assay using endogenous human protein. In this assay, recombinant GST-PS1-Loop or GST-PS1-Loop FP/VS/AA proteins were incubated with glutathione agarose beads, the binding buffer was allowed to flow through the column before an equivalent amount of protein from HEK293T cell lysate (50 mg) was added to the columns. The columns were incubated overnight at 4°C with the cell lysate. The following morning the unbound lysate was allowed to flow through the column and the beads were then washed 4 times in binding buffer. After washing the beads were collected in an Eppendorf tube and boiled in 500 ml of 2X Laemmli sample buffer. Samples taken at each stage of the pulldown assay were separated by SDS-PAGE and proteins were detected by coomassie staining **(Figures 5.1B and 5.1C)** showing

the intensity and size-diversity of proteins that bound to the GST-PS1-Loop **(Figure 5.1B lane 9)** and GST-PS1-Loop-FP/VS/AA recombinant proteins **(Figure 5.1C lane 9)**. These gels demonstrate that this experimental approach can be used to investigate PS1 CUE domain function as it demonstrates that the recombinant proteins interact with a range of proteins of different sizes.

5.2 Endogenous Rab11 is purified by recombinant GST-PS1 Loop Proteins

To validate this pulldown assay it was decided to determine if any known PS1 intracellular loop domain interacting proteins were pulled out of HEK293T lysate by the GST-PS1 Loop and GST-PS1 Loop FP/VS/AA recombinant proteins. Rab11 is a known PS1 interacting protein that interacts with the intracellular loop domain of PS1 [Dumanchin et al 1999] and this protein was used to validate the assay. Protein samples taken during the GST-pulldown assay were separated by SDS-PAGE and then immunoblotted with an anti-Rab11 antibody. Rab11 can be seen to be pulled down by the recombinant GST-PS1 Loop **(Figure 5.2A)** and GST-PS1 Loop FP/VS/AA proteins **(Figure 5.2B)**. The blot was also probed with anti-PS1 CTF antibody to show the presence of the recombinant GST-tagged proteins. These data further validates the GST-pulldown assay for the purpose of investigating PS1 intracellular loop interacting proteins.

5.3 The Deubiquitinase Inhibitor Phenanthroline Prevents GST Binding To Glutathione Agarose Beads

Sites of ubiquitination can be identified through mass spectrometry by the presence of the GGK motif in analysed tryptic peptide [Shi *et al* 2011]. This motif represents

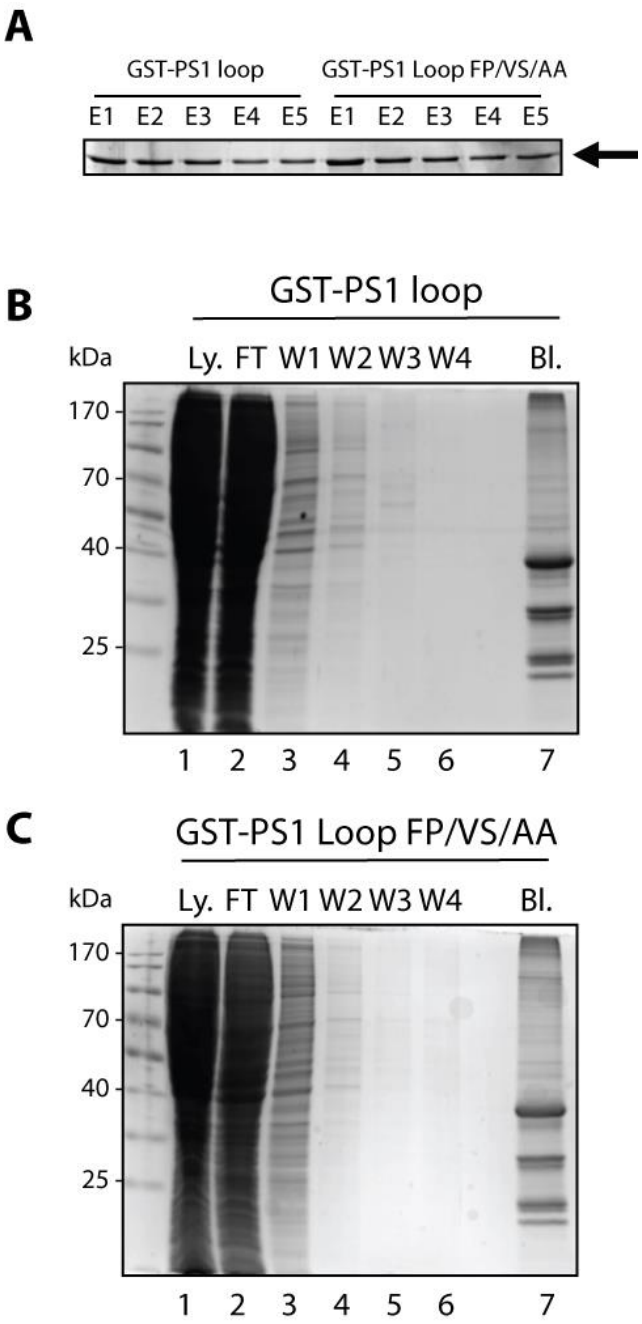


Figure 5.1 GST-PS1 Loop pulldown from endogenous HEK293T cells. (A) GST-PS1-Loop and GST-PS1-Loop FP/VS/AA proteins were purified from BL21 E. coli cells. The purified proteins were collected into 5 elutions and indicated above with an arrow. Equivalent protein purification was demonstrated by separation by SDS-PAGE and coomassie staining. Cell lsyates from HEK293T cells were incubated with (B) GST-PS1 Loop or (C) GST-PS1 Loop FP/VS/AA recombinant proteins bound to glutathione-agarose beads. The unbound proteins were allowed to flow through the column before the beads were washed 4 times. Bound proteins were removed from the bead bed through boiling. Protein samples were separated by SDS-PAGE on 12% gels and proteins were detected by coomassie staining. E: elution; kDa: kilodaltons; Ly: lysate; FT: flow through; W: wash; Bl: boil.

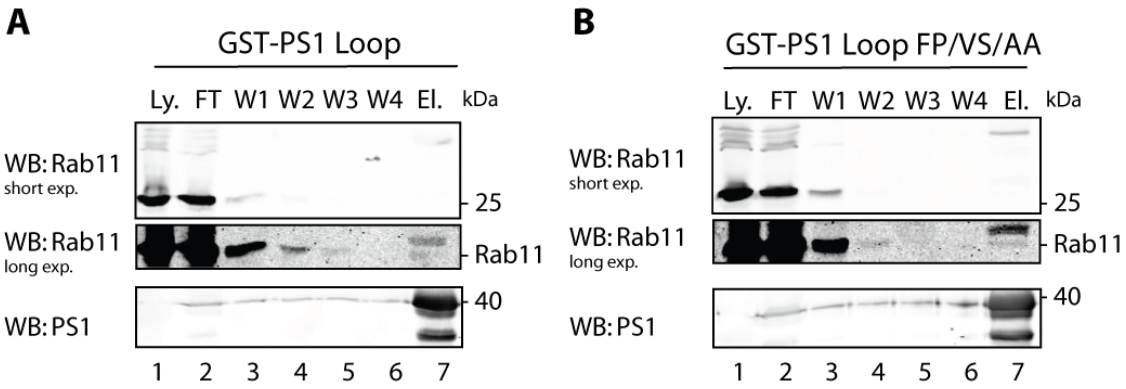


Figure 5.2 Western blot analysis of GST-PS1 Loop pulldown from HEK293T cells. Cell lsyates from HEK293T cells were incubated with (A) GST-PS1 Loop or (B) GST-PS1 Loop FP/VS/AA recombinant proteins bound to glutathione-agarose beads. The unbound proteins were allowed to flow through the column before the beads were washed 4 times. Bound proteins were removed from the bead bed through boiling. Protein samples taken at different stages through the assay were separated by SDS-PAGE on 12% gels and were subject to immunoblotting with either anti-Rab11 or anti-PS1 CTF antibodies. WB: western blot; kDa: kilodalton; Ly: lysate; FT: flow through; W: wash; El: Elution.

the final two glycine residues of ubiquitin and the lysine residue on which they are covalently bound. To find these sites of ubiquitination it is necessary to inhibit deubiquitinase (DUB) proteins when lysing the cells that will be analysed. As iodoacetamide has been known to cause the formation of pseudo-ubiquitinated sites it was decided to test the DUB inhibitor phenanthroline (Phen) on the GST-pulldown assay [Shi *et al* 2011]. GST, GST-PS1-Loop or GST-PS1-Loop FP/VS/AA proteins were incubated with glutathione agarose beads, the binding buffer was allowed to flow through the column before an equivalent amount of protein from HEK293T cells lysed in binding buffer supplemented with 8 mM phenanthroline was added to the column. The column was incubated overnight at 4°C with the cell lysate. The following morning the unbound lysate was allowed to flow through the column and the beads were then washed 4 times in binding buffer. After washing the bound protein was eluted from the column with 50 μM glutathione elution buffer. The bound proteins were eluted from the beads in this assay rather than boiled to try and minimise non-specific protein binding. Samples were retained at each stage of the assay, then separated by SDS-PAGE and protein binding was detected by coomassie staining of each gel. The recombinant GST protein is not clearly visible after the binding process suggesting that the phenanthroline may cause GST to detach from the glutathione-agarose beads **(lane 9 of Figure 5.3A)**. To test this hypothesis the experiment was repeated using GST-PS1-Loop and GST-PS1-Loop FP/VS/AA proteins. Unbound GST-PS1-Loop and GST-PS1-Loop FP/VS/AA proteins and samples of the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA bound beads were retained prior to the addition of the HEK293T lysate. The HEK293T cells were still lysed in binding buffer supplemented with the DUB inhibitor phenanthroline (8 mM). The GST-PS1-Loop and GST-PS1-Loop FP/VS/AA proteins were bound to

the glutathione agarose beads prior to incubation with the HEK293T cell lysate **(Lanes 1 of Figures 5.3B and 5.3C)**. However, no equivalent bands to these recombinant proteins can be seen on the gels after incubation with the phenanthroline supplemented binding buffer. As the phenanthroline appeared to be causing the GST-tagged proteins to detach from the glutathione agarose beads it was decided to leave the DUB inhibitor out of the GST-pulldown of endogenous human protein assay.

5.4 GST-Pulldown of Endogenous Human Proteins for Mass spectrometry Analysis

Once the GST-pulldown assay had been optimised the samples for mass spectrometry analysis were prepared. Recombinant GST, GST-PS1-Loop or GST-PS1-Loop FP/VS/AA proteins were incubated with glutathione agarose beads prior to incubation with an equivalent amount of HEK293T lysate (50 mg per experiment). The recombinant proteins were incubated with the HEK293T lysate overnight at 4°C, unbound protein was allowed to flow through the column and the bead bed was washed 4 times with binding buffer. For the GST sample **(Figure 5.4A)** the bound proteins were first eluted from the beads using a 50 mM elution buffer but as can be seen in lane 10 of the gel (marked 'GST') that the recombinant GST protein is not clearly visible after elution. After consultation with Fingerprints Proteomics (Dundee, UK), who performed our mass spectrometry analysis, it was decided to remove bound protein from the beads through boiling in 2X Laemmli sample buffer as a means of increasing protein concentration in the sample. Here the GST protein appears as a much stronger band (marked 'GST') and other bound proteins can also be seen clearly on the gel **(Figure 5.4B)**. For the GST-PS1-Loop **(Figure 5.4C)** and GST-PS1-Loop FP/VS/AA **(Figure 5.4D)** samples the same

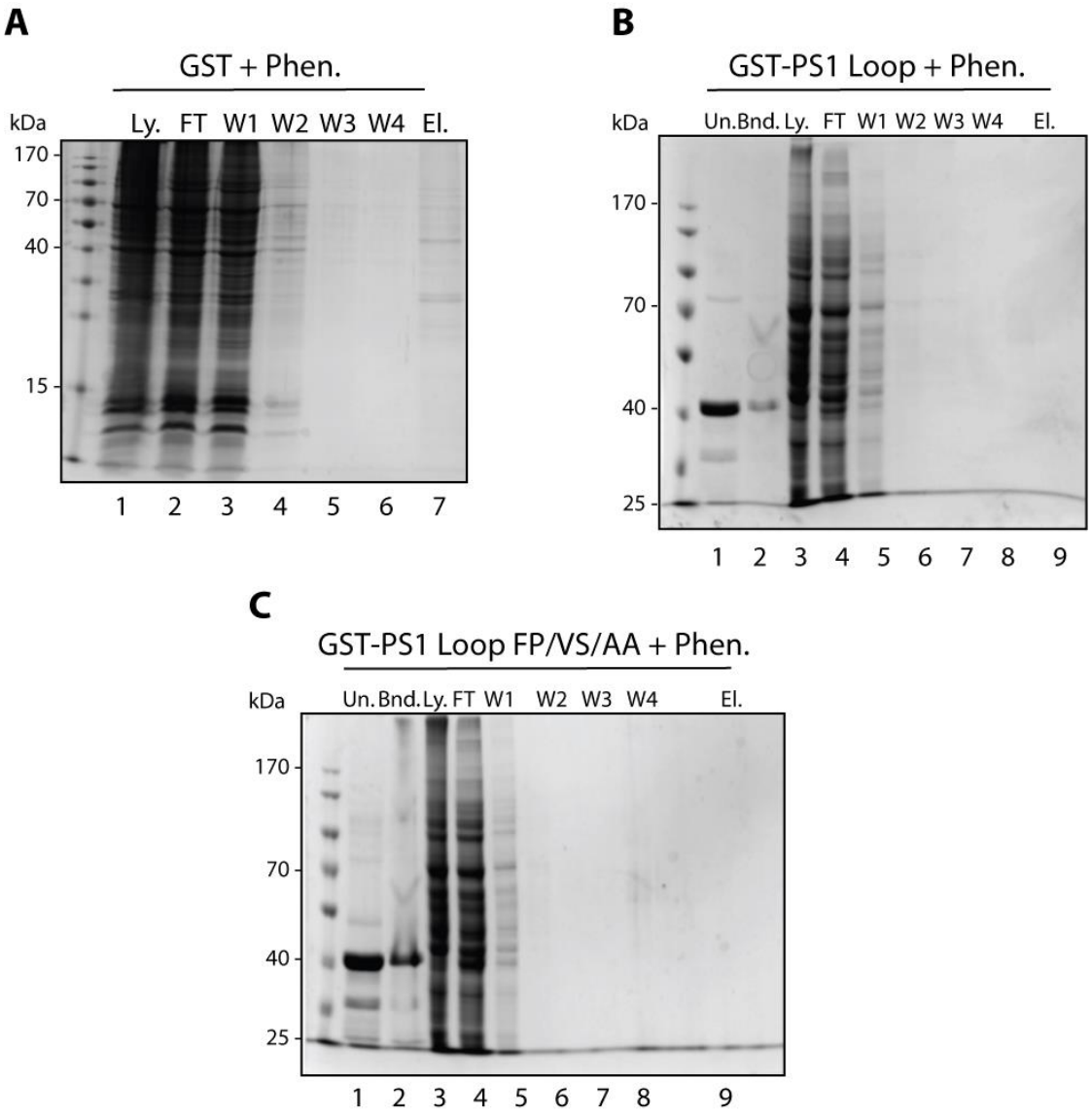


Figure 5.3 Phenanthroline inhibits binding of recombinant GST-tagged proteins to glutathione agarose beads. HEK293T cells were lysed in binding buffer supplemented with 8 mM phenanthroline (PTL). The HEK293T lysates were incubated with (A) GST, (B) GST-PS1 Loop or (C) GST-PS1 Loop FP/VS/AA recombinant proteins bound to glutathione-agarose beads. The unbound proteins were allowed to flow through the column before the beads were washed 4 times. Bound proteins were removed from the bead bed through elution with 50 mM glutathione elution buffer. Protein samples were separated by SDS-PAGE and bound proteins were detected by Coomassie staining. El: elution; kDa: kilodaltons; Ly: lysate; FT: flow through; W: wash; Un: unbound; Bnd: bound.

process was repeated and bound proteins were removed from the beads by boiling in 2X Laemmli sample buffer andhere the recombinant proteins are marked as 'GST-PS1-Loop' and 'GST-PS1-Loop FP/VS/AA' **(the final lanes of Figures 5.4C and 5.4D)**. These samples were adjudged to be of sufficient quality and concentration to be used for mass spectrometry analysis.

5.5 Mass Spec Identification of Pulled Down Proteins

Three samples; GST, GST-PS1-Loop and GST-PS1-Loop FP/VS/AA were sent for mass spectrometry analysis. The GST sample was acting as a negative control for the experiment; proteins that were identified in this sample would be excluded from consideration if also present either of the other two samples. Bound proteins were separated by SDS-PAGE under laminar air flow conditions to reduce contamination (see **Figure 5.5** for image of sample separation), gel purified and then subject to tryptic digestion as previously described [Walsh *et al* 2002]. Tryptic peptides from the samples were analysed using matrix–assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The analysed tryptic peptides were identified and scored using the Mascot server (www.matrixscience.com). In the Mascot server each analysed peptide will be assigned an ion score based on the probability that that the calculated mass of a peptide matches that of a queried protein in the Mascot database. The Mascot score represents the sum of the ion scores that a protein identified through a mass spectrometry analysis accrues i.e. a protein will achieve a higher Mascot score when there is a greater number of unique peptides analysed by the mass spectrometry machine. Therefore, the higher the Mascot score that an identified protein achieves the more confident you can be that this is a correctly identified protein. The proteins identified in each of the three samples were compiled **(Table 5.1)** showing both the total number of proteins

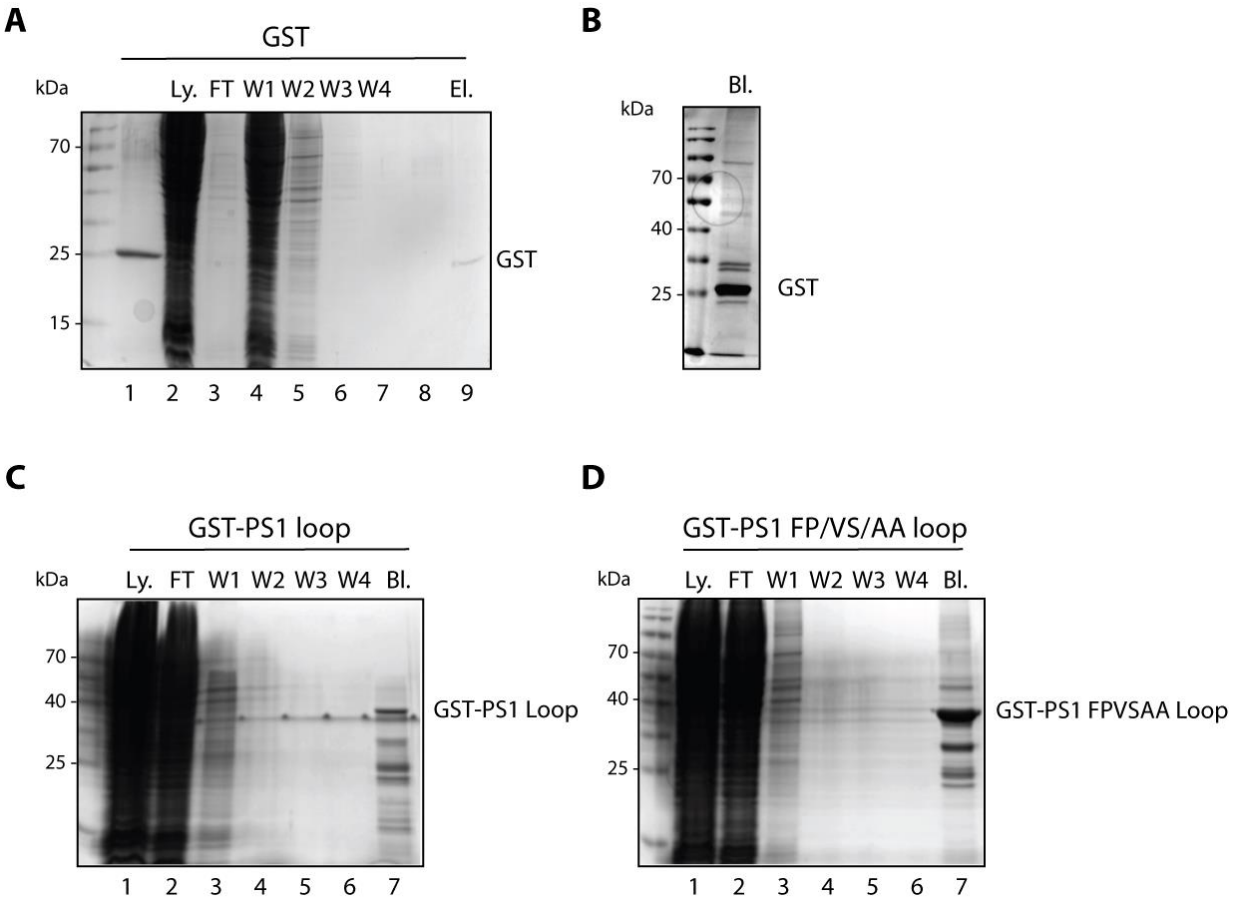


Figure 5.4 GST-pulldown of endogenous HEK293T proteins for mass spectrometry analysis. HEK293T lysates were incubated with (A) and (B) GST, (C) GST-PS1-Loop or (D) GST-PS1-Loop FP/VS/AA recombinant proteins bound to glutathione-agarose beads. The unbound proteins were allowed to flow through the column before the beads were washed 4 times. Bound proteins were removed from the bead bed through either (A) elution with 50 mM glutathione buffer or (B-D) through boiling. Bound proteins were separated by SDS-PAGE on 12% gels and proteins were detected by Coomassie staining. Bl: Boil El: elution; kDa: kilodaltons; Ly: lysate; FT: flow through; W: wash; Un: unbound; Bnd: bound.

identified and the total number of proteins through mass spectrometry analysis and the number of proteins remaining after the cutoff was applied.

Table 5.1 Total number of proteins identified in mass spectrometry analysis of GST, GST-PS1 Loop and GST-PS1 Loop FP/VS/AA samples and number of proteins remaining after application of cut-off.

Data Set	Total No. of proteins	No. of Proteins after Cut-off
GST	584	264
GST-PS1 Loop	380	162
GST-PS1 Loop FP/VS/AA	353	172

The identified proteins from the GST, GST-PS1-Loop and GST-PS1-Loop FP/VS/AA samples are presented in full in Appendices 7.1-7.3. The mass spectrometry results for the three samples are presented in Table 5.1 showing the total amount of proteins identified per sample and the amount of proteins remaining after a cut-off had been applied to each sample list. The cut-off was applied to any protein that had only 1 unique peptide identified through the mass spectrometry analysis [Atrih *et al* 2014]. This removed 320 proteins from the GST dataset, 218 from the GST-PS1-Loop dataset and GST-PS1-Loop FP/VS/AA dataset.

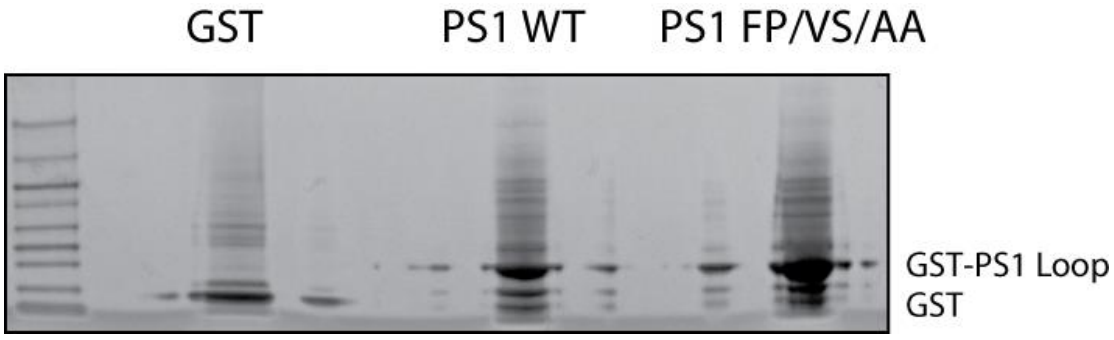


Figure 5.5 Separation of samples for mass spec analysis. Prior to mass spectrometry analysis protein samples were separated by SDS-PAGE under laminar airflow conditions and proteins were detected by coomassie staining. The GST (GST), GST-PS1 Loop (PS1 WT) and GST-PS Loop FP/VS/AA (PS1 FP/VS/AA) samples are highlighted on top of the panel. The GST and GST-PS1 Loop recombinant proteins are highlighted on the right of the panel.

5.6 Alignment of Mass spectrometry PS1 Tryptic Peptides

Our hypothesis for the function of the PS1 CUE domain was that it was involved in regulating either intermolecular PS1 protein-protein interactions or intramolecular PS1-PS1 interactions. The GST-PS1-Loop recombinant proteins contain the intracellular loop domain of PS1 that is present between residues 265 and 380 of PS1. By aligning the sequences of the PS1 tryptic peptides identified with the primary sequence of the PS1 holoprotein the presence of any tryptic peptides that originate from endogenous PS1 protein can be detected. Endogenous proteins can be detected if any tryptic PS1 peptides that align with PS1 sequences outside of the intracellular loop of PS1. The unique PS1 peptides identified in the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA samples were aligned with the primary sequence of PS1 using AlignX. All peptides examined aligned between residues 270 and 380 of the PS1 primary sequence **(Figure 5.6)**. As all PS1 tryptic peptides aligned with the intracellular loop domain of PS1 these data suggests that the intracellular loop domain of PS1, and by extension the PS1 CUE domain, is not involved in intramolecular PS1-PS1 interactions. This suggests that the PS1 CUE domain is involved in the regulation of intermolecular PS1 protein-protein interactions.

```
Presenilin 1 (270-319)          MLVETAQERNETLFPALIYSSTMVWLVNMAEGDPEAQRRVSKNSKYNAES
GST-PS1 peptide 1               --------------------------------------------------
GST-PS1 peptide 2               --------------------------------------------------
GST-PS1 peptide 3               ----------------------------------------------YNAES
GST-PS1 peptide 4               MLVETAQER-----------------------------------------
GST-PS1 peptide 5               --------------------------------------------------
GST-PS1-peptide 6               --------------------------------------------------
GST-PS1 peptide 7               ----------------------------------------------YNAES
GST-PS1 peptide 8               MLVETAQER-----------------------------------------
GST-PS1 peptide 9               ----------------------------------------------YNAES
GST-PS1 FPVSAA peptide 1        --------------------------------------------------
GST-PS1 FPVSAA peptide 2        --------------------------------------------------
GST-PS1 FPVSAA peptide 3        --------------------------------------------------
GST-PS1 FPVSAA peptide 5        MLVETAQER-----------------------------------------
GST-PS1 FPVSAA peptide 4        ----------------------------------------------YNAES
GST-PS1 FPVSAA peptide 6        ----------------------------------------------YNAES
GST-PS1 FPVSAA peptide 7        --------------------------------------------------
GST-PS1 FPVSAA peptide 8        --------------------------------------------------
GST-PS1 FPVSAA peptide 9        --------------------------------------------------
GST-PS1 FPVSAA peptide 10       ------------------------------------------NSKYNAES

Presenilin 1 (320-369)          TERESQDTVAENDDGGFSEEWEAQRDSHLGPHRSTPESRAAVQELSSSIL
GST-PS1 peptide 1               ------------------------------------------AAVQELSSSIL
GST-PS1 peptide 2               ---ESQDTVAENDDGGFSEEWEAQR-------------------------
GST-PS1 peptide 3               TERESQDTVAENDDGGFSEEWEAQR-------------------------
GST-PS1 peptide 4               --------------------------------------------------
GST-PS1 peptide 5               ------------------------------------------AAVQELSSSIL
GST-PS1 peptide 6               ---ESQDTVAENDDGGFSEEWEAQRDSHLGPHR-----------------
GST-PS1 peptide 7               TER-----------------------------------------------
GST-PS1 peptide 8               --------------------------------------------------
GST-PS1 peptide 9               TERESQDTVAENDDGGFSEEWEAQRDSHLGPHR-----------------
GST-PS1 FPVSAA peptide 1        ------------------------------------------AAVQELSSSIL
GST-PS1 FPVSAA peptide 2        ---ESQDTVAENDDGGFSEEWEAQR-------------------------
GST-PS1 FPVSAA peptide 3        ------------------------------------------AAVQELSSSIL
GST-PS1 FPVSAA peptide 5        --------------------------------------------------
GST-PS1 FPVSAA peptide 4        TERESQDTVAENDDGGFSEEWEAQR-------------------------
GST-PS1 FPVSAA peptide 6        TER-----------------------------------------------
GST-PS1 FPVSAA peptide 7        ---ESQDTVAENDDGGFSEEWEAQRDSHLGPHR-----------------
GST-PS1 FPVSAA peptide 8        ----------------------------------STPESRAAVQELSSSIL
GST-PS1 FPVSAA peptide 9        ------------------------------------------AAVQELSSSIL
GST-PS1 FPVSAA peptide 10       TER-----------------------------------------------

Presenilin 1 (370-380)          AGEDPEERGVK
GST-PS1 peptide 1               AGEDPEER---
GST-PS1 peptide 2               -----------
GST-PS1 peptide 3               -----------
GST-PS1 peptide 4               -----------
GST-PS1 peptide 5               AGEDPEERGVK
GST-PS1 peptide 6               -----------
GST-PS1 peptide 7               -----------
GST-PS1 peptide 8               -----------
GST-PS1 peptide 9               -----------
GST-PS1 FPVSAA peptide 1        AGEDPEER---
GST-PS1 FPVSAA peptide 2        -----------
GST-PS1 FPVSAA peptide 3        AGEDPEER---
GST-PS1 FPVSAA peptide 5        -----------
GST-PS1 FPVSAA peptide 4        -----------
GST-PS1 FPVSAA peptide 6        -----------
GST-PS1 FPVSAA peptide 7        -----------
GST-PS1 FPVSAA peptide 8        AGEDPEER---
GST-PS1 FPVSAA peptide 9        AGEDPEERGVK
GST-PS1 FPVSAA peptide 10       -----------
```

Figure 5.6 Alignment of PS1 tryptic peptides with the primary sequence of PS1 holoprotein. The tryptic peptides identified by the mass spectrometry analysis of the GST-PS1-Loop and GST-Ps1-Loop FP/VS/AA samples were aligned with the primary sequence of PS1 using AlignX. All peptides examined aligned with PS1 between residues 270-280.

5.7 Analysis of Proteins Identified Through Mass spectrometry Analysis

After the cut-off had been applied to all three datasets, next the proteins identified in the negative control GST dataset were removed from both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datatsets. Any proteins identified in the negative control sample are likely to bind to either the glutathione-agarose beads or the GST protein and as such represent non-specific binding in either the GST-PS1-Loop or GST-PS1-Loop FP/VS/AA samples. While PS1 was the highest scoring protein for both the GST-PS1-Loop (13124.15) and GST-PS1-Loop FP/VS/AA datasets (23106.01), it also received a Mascot score in the GST dataset (370.42) suggesting that a small amount of endogenous PS1 interacted with the negative control sample. The highest Mascot scoring protein that remained after removal of the negative control dataset was δ-catenin for both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA samples. Plakophilin-4 also received a high Mascot score in both protein samples. Both proteins have previously been shown to interact with PS1 which demonstrates the validity of these results [Tanahashi *et al* 1999; Stahl *et al* 1999]. The other identified proteins remaining after removal of the negative control have been tabulated into three separate tables. Table 5.2 records the bound proteins that were identified in both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA samples. The table gives the accession code and name of the proteins, the percentage of the proteins sequence covered by the examined peptides, the Mascot score received and the number of proteins, peptides and unique peptides recorded by the mass spectrometry analysis. The same details are presented for proteins unique to the GST-PS1-Loop sample in Table 5.3 and for the GST-PS1-Loop FP/VS/AA sample in Table 5.4.

Table 5.2 Common bound proteins identified through mass spectrometry analysis of GST-PS1 Loop and GST-PS1 Loop FP/VS/AA sample.

Proteins from GST-PS1 Loop sample are highlighted, while proteins from GST-PS1 Loop FP/VS/AA are unhighlighted.

Accession	Protein name	Mascot Score	Coverage	# Proteins	# Unique Peptides	# Peptides
O60716	Catenin delta-1 OS=Homo sapiens GN=CTNND1	3890.51	47.93	1	38	38
	Catenin delta-1 OS=Homo sapiens GN=CTNND1	3953.12	55.06	1	41	41
Q99569	Plakophilin-4 OS=Homo sapiens GN=PKP4	670.78	18.20	2	18	18
	Plakophilin-4 OS=Homo sapiens GN=PKP4	692.53	20.30	2	17	17
P68371	Tubulin beta-4B chain OS=Homo sapiens GN=TUBB4B	1269.99	42.70	4	2	14
	Tubulin beta-4B chain OS=Homo sapiens GN=TUBB4B	1194.15	48.09	5	2	18
P35579	Myosin-9 OS=Homo sapiens GN=MYH9	322.08	5.71	3	8	8
	Myosin-9 OS=Homo sapiens GN=MYH9	172.57	3.32	3	4	5
Q9BUF5	Tubulin beta-6 chain OS=Homo sapiens GN=TUBB6	491.59	14.35	2	1	6
	Tubulin beta-6 chain OS=Homo sapiens GN=TUBB6	539.27	22.87	2	3	10
O76031	ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial OS=Homo sapiens GN=CLPX	192.54	8.06	1	4	4
	ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial OS=Homo sapiens GN=CLPX	321.58	20.22	1	9	9
Q96EY1	DnaJ homolog subfamily A member 3, mitochondrial OS=Homo sapiens GN=DNAJA3	172.14	10.21	1	4	4
	DnaJ homolog subfamily A member 3, mitochondrial OS=Homo sapiens GN=DNAJA3	132.15	14.79	1	5	5
Q8NI60	Chaperone activity of bc1 complex-like, mitochondrial OS=Homo sapiens GN=ADCK3	143.90	7.57	1	4	4
	Chaperone activity of bc1 complex-like, mitochondrial OS=Homo sapiens GN=ADCK3	139.88	7.73	1	4	4
Q9P2J5	Leucine--tRNA ligase,	183.48	3.23	1	3	3

	cytoplasmic OS=Homo sapiens GN=LARS					
	Leucine--tRNA ligase, cytoplasmic OS=Homo sapiens GN=LARS	146.02	2.38	1	2	2
P35580	Myosin-10 OS=Homo sapiens GN=MYH10	179.70	2.23	1	3	3
	Myosin-10 OS=Homo sapiens GN=MYH10	112.09	1.77	1	2	3
P11310	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Homo sapiens GN=ACADM	117.07	8.31	1	3	3
	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Homo sapiens GN=ACADM	291.92	16.39	1	5	5
P62424	60S ribosomal protein L7a OS=Homo sapiens GN=RPL7A	115.40	12.41	1	3	3
	60S ribosomal protein L7a OS=Homo sapiens GN=RPL7A	98.25	9.02	1	2	2
P33993	DNA replication licensing factor MCM7 OS=Homo sapiens GN=MCM7	106.36	6.68	1	3	3
	DNA replication licensing factor MCM7 OS=Homo sapiens GN=MCM7	173.67	11.27	1	6	6
P40429	60S ribosomal protein L13a OS=Homo sapiens GN=RPL13A	99.78	14.29	2	3	3
	60S ribosomal protein L13a OS=Homo sapiens GN=RPL13A	102.39	14.29	2	3	3
Q9Y2S7	Polymerase delta-interacting protein 2 OS=Homo sapiens GN=POLDIP2	94.87	10.87	1	3	3
	Polymerase delta-interacting protein 2 OS=Homo sapiens GN=POLDIP2	227.43	18.21	1	5	5
P12236	ADP/ATP translocase 3 OS=Homo sapiens GN=SLC25A6	91.61	10.40	3	1	3
	ADP/ATP translocase 3 OS=Homo sapiens GN=SLC25A6	70.71	5.70	3	1	2
P31689	DnaJ homolog subfamily A member 1 OS=Homo sapiens GN=DNAJA1	91.06	11.59	3	3	3
	DnaJ homolog subfamily A member 1 OS=Homo sapiens GN=DNAJA1	185.04	17.88	6	4	5
P48047	ATP synthase subunit O, mitochondrial OS=Homo sapiens GN=ATP5O	175.95	13.15	1	2	2
	ATP synthase subunit O, mitochondrial OS=Homo sapiens GN=ATP5O	127.51	18.31	1	3	3
P84098	60S ribosomal protein L19 OS=Homo sapiens GN=RPL19	143.49	13.27	1	2	2
	60S ribosomal protein L19 OS=Homo sapiens GN=RPL19	106.96	13.27	1	2	2
P13674	Prolyl 4-hydroxylase subunit alpha-1 OS=Homo sapiens	142.05	5.24	1	2	2

	GN=P4HA1					
	Prolyl 4-hydroxylase subunit alpha-1 OS=Homo sapiens GN=P4HA1	161.05	8.05	1	3	3
P40938	Replication factor C subunit 3 OS=Homo sapiens GN=RFC3	102.17	7.87	1	2	2
	Replication factor C subunit 3 OS=Homo sapiens GN=RFC3	114.73	7.87	1	2	2
Q99729	Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB	87.16	6.33	1	2	2
	Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB	70.69	6.33	1	2	2
Q9Y3Z3	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1 OS=Homo sapiens GN=SAMHD1	82.07	3.99	1	2	2
	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1 OS=Homo sapiens GN=SAMHD1	154.00	10.86	1	5	5
Q96I99	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial OS=Homo sapiens GN=SUCLG2	81.43	3.94	1	2	2
	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial OS=Homo sapiens GN=SUCLG2	249.84	15.28	1	6	6
Q96C36	Pyrroline-5-carboxylate reductase 2 OS=Homo sapiens GN=PYCR2	70.43	6.56	1	2	2
	Pyrroline-5-carboxylate reductase 2 OS=Homo sapiens GN=PYCR2	120.98	9.38	1	2	2

Table 5.3 Proteins identified through mass spectrometry analysis unique to the GST-PS1 Loop dataset.

Accession	Protein name	Mascot Score	Coverage	# Proteins	# Unique Peptides	# Peptides
P04259	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B	619.71	32.09	6	1	16
P63261	Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1	909.76	44.53	7	1	13
P09488	Glutathione S-transferase Mu 1 OS=Homo sapiens GN=GSTM1	809.33	24.31	2	1	7
P63267	Actin, gamma-enteric smooth muscle OS=Homo sapiens GN=ACTG2	212.41	19.41	5	1	7
P13646	Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13	160.66	9.39	4	1	5
O75828	Carbonyl reductase [NADPH] 3 OS=Homo sapiens GN=CBR3	1112.77	19.86	1	1	4
Q9NR30	Nucleolar RNA helicase 2 OS=Homo sapiens GN=DDX21	150.55	7.02	1	4	4
Q86Y46	Keratin, type II cytoskeletal 73 OS=Homo sapiens GN=KRT73	127.99	6.48	4	1	4
Q86V81	THO complex subunit 4 OS=Homo sapiens GN=ALYREF	225.81	21.40	1	3	3
P12277	Creatine kinase B-type OS=Homo sapiens GN=CKB	153.35	12.34	1	3	3
P05386	60S acidic ribosomal protein P1 OS=Homo sapiens GN=RPLP1	118.58	20.18	2	2	2
P13637	Sodium/potassium-transporting ATPase subunit alpha-3 OS=Homo sapiens GN=ATP1A3	115.31	3.26	4	2	2
P62750	60S ribosomal protein L23a OS=Homo	95.35	14.10	1	2	2

	sapiens GN=RPL23A					
Q8N1N4	Keratin, type II cytoskeletal 78 OS=Homo sapiens GN=KRT78	95.04	3.85	2	1	2
Q9ULV4	Coronin-1C OS=Homo sapiens GN=CORO1C	90.15	5.70	1	2	2
O00483	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4 OS=Homo sapiens GN=NDUFA4	85.65	27.16	1	2	2
P62826	GTP-binding nuclear protein Ran OS=Homo sapiens GN=RAN	81.61	10.19	1	2	2
P08621	U1 small nuclear ribonucleoprotein 70 kDa OS=Homo sapiens GN=SNRNP70	80.54	4.35	1	2	2
P68431	Histone H3.1 OS=Homo sapiens GN=HIST1H3A	74.48	11.76	5	2	2
P27348	14-3-3 protein theta OS=Homo sapiens GN=YWHAQ	71.43	7.35	7	2	2
Q9BUQ8	Probable ATP-dependent RNA helicase DDX23 OS=Homo sapiens GN=DDX23	62.71	2.80	1	2	2
Q8IX12	Cell division cycle and apoptosis regulator protein 1 OS=Homo sapiens GN=CCAR1	55.33	1.57	1	2	2

Table 5.4 Proteins identified through mass spectrometry analysis unique to the GST-PS1 Loop FP/VS/AA dataset.

Accession	Protein name	Score	Coverage	# Proteins	# Unique Peptides	# Peptides
P46459	Vesicle-fusing ATPase OS=Homo sapiens GN=NSF	243.78	10.75	1	7	7
Q9UKB1	F-box/WD repeat-containing protein 11 OS=Homo sapiens GN=FBXW11	240.52	18.27	2	6	6
Q92947	Glutaryl-CoA dehydrogenase, mitochondrial OS=Homo sapiens GN=GCDH	199.93	18.95	1	4	4
O60762	Dolichol-phosphate mannosyltransferase subunit 1 OS=Homo sapiens GN=DPM1	150.18	23.08	1	4	4
Q7Z7L1	Schlafen family member 11 OS=Homo sapiens GN=SLFN11	134.32	5.88	1	4	4
P30837	Aldehyde dehydrogenase X, mitochondrial OS=Homo sapiens GN=ALDH1B1	130.82	8.51	1	4	4
P11172	Uridine 5'-monophosphate synthase OS=Homo sapiens GN=UMPS	148.10	7.92	1	3	3
P32322	Pyrroline-5-carboxylate reductase 1, mitochondrial OS=Homo sapiens GN=PYCR1	113.06	14.42	1	3	3
P0C0S5	Histone H2A.Z OS=Homo sapiens GN=H2AFZ	112.94	20.31	5	1	3
P53597	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial OS=Homo sapiens GN=SUCLG1	148.30	3.76	1	2	2
P22695	Cytochrome b-c1 complex subunit 2, mitochondrial OS=Homo sapiens GN=UQCRC2	131.37	6.62	1	2	2
Q9HAV7	GrpE protein homolog 1, mitochondrial OS=Homo sapiens GN=GRPEL1	113.90	12.44	1	2	2
Q99714	3-hydroxyacyl-CoA dehydrogenase type-2 OS=Homo sapiens GN=HSD17B10	107.86	9.58	1	2	2
Q7Z3Y8	Keratin, type I cytoskeletal 27 OS=Homo sapiens GN=KRT27	106.48	4.58	4	1	2
Q14203	Dynactin subunit 1 OS=Homo sapiens GN=DCTN1	105.87	1.41	4	1	2
Q8NE71	ATP-binding cassette sub-family F member 1 OS=Homo sapiens GN=ABCF1	103.95	3.08	1	2	2
P07686	Beta-hexosaminidase subunit beta OS=Homo sapiens GN=HEXB	92.49	4.32	1	2	2
Q16531	DNA damage-binding protein 1 OS=Homo sapiens GN=DDB1	90.68	1.75	1	2	2
P23258	Tubulin gamma-1 chain OS=Homo sapiens GN=TUBG1	90.25	11.09	2	2	2
Q8N4T8	Carbonyl reductase family member 4 OS=Homo sapiens GN=CBR4	86.74	10.13	1	2	2

P34897	Serine hydroxymethyltransferase, mitochondrial OS=Homo sapiens GN=SHMT2	82.59	4.37	1	2	2
Q5JPH6	Probable glutamate--tRNA ligase, mitochondrial OS=Homo sapiens GN=EARS2	77.55	4.21	1	2	2
P15924	Desmoplakin OS=Homo sapiens GN=DSP	76.92	0.77	1	2	2
Q16775	Hydroxyacylglutathione hydrolase, mitochondrial OS=Homo sapiens GN=HAGH	74.71	7.79	1	2	2
P81605	Dermcidin OS=Homo sapiens GN=DCD	73.48	22.73	1	2	2
Q15120	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 3, mitochondrial OS=Homo sapiens GN=PDK3	72.91	4.93	1	2	2
Q9UN37	Vacuolar protein sorting-associated protein 4A OS=Homo sapiens GN=VPS4A	72.87	4.35	3	2	2
Q9NTJ3	Structural maintenance of chromosomes protein 4 OS=Homo sapiens GN=SMC4	72.65	1.71	1	2	2
O60884	DnaJ homolog subfamily A member 2 OS=Homo sapiens GN=DNAJA2	67.93	7.77	1	2	2
Q53H96	Pyrroline-5-carboxylate reductase 3 OS=Homo sapiens GN=PYCRL	65.76	8.03	1	2	2
Q9UL03	Integrator complex subunit 6 OS=Homo sapiens GN=INTS6	59.52	2.14	2	2	2

5.8 PANTHER System of Protein Classification

The lists of proteins identified in both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA samples were first reduced in size by the application of a cut-off with the removal of proteins that were identified by a single peptide. After this the lists were further reduced by the removal of any protein that was also contained with the negative control GST sample. This reduced the number of proteins identified in the GST-PS1-Loop sample to 48 from the initial size of 380 and reduced the GST-PS1-Loop FP/VS/AA sample to 57 from an initial size of 353. These proteins are compiled into the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets that are presented in Table 5.5. These datasets were then used to investigate the function of PS1 CUE domain using bioinformatics tools. These bioinformatics tools were used to look for any relevant differences in the biological function of proteins contained in either dataset.

To do this protein analysis through evolutionary relationships (PANTHER) classification system was used (www.pantherdb.org) [Mi *et al* 2012]. PANTHER analyses submitted lists of protein accession numbers or gene names and categorises them using the Gene Ontology annotations molecular function, biological process or pathway [The Gene Ontology Consortium 2011]. The molecular function category, defines proteins by their direct function e.g. kinases, phosphatases etc.; over 9000 different molecular function terms were used as of 2011. The GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets were split into 6 different molecular function categories: translation regulator activity, binding, enzyme regulator activity, structural molecule activity, catalytic activity and transporter activity. The molecular function of the datasets are compared in absolute terms using bar chart **(Figure** .

Table 5.5 List of gene and proteins names contained within the GST-PS1-Loop or GST-PS1-Loop FP/VS/AA datasets.

GST-PS1-Loop Dataset		GST-PS1-Loop FP/VS/AA Dataset	
Gene Name	**Protein name**	**Gene Name**	**Protein name**
PSEN1	Presenilin-1	PSEN1	Presenilin-1
CTNND1	Catenin delta-1	CTNND1	Catenin delta-1
TUBB4B	Tubulin beta-4B chain	TUBB4B	Tubulin beta-4B chain
CBR3	Carbonyl reductase [NADPH] 3	PKP4	Plakophilin-4
ACTG1	Actin, cytoplasmic 2	TUBB6	Tubulin beta-6 chain
GSTM1	Glutathione S-transferase Mu 1	CLPX	ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial
PKP4	Plakophilin-4	ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial
KRT6B	Keratin, type II cytoskeletal 6B	SUCLG2	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial
TUBB6	Tubulin beta-6 chain	NSF	Vesicle-fusing ATPase/NSF
MYH9	Myosin-9	FBXW11	F-box/WD repeat-containing protein 11
ALYREF	THO complex subunit 4	POLDIP2	Polymerase delta-interacting protein 2
ACTG2	Actin, gamma-enteric smooth muscle	GCDH	Glutaryl-CoA dehydrogenase, mitochondrial
CLPX	ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial	DNAJA1	DnaJ homolog subfamily A member 1
LARS	Leucine--tRNA ligase, cytoplasmic	MCM7	DNA replication licensing factor MCM7
MYH10	Myosin-10	MYH9	Myosin-9
ATP5O	ATP synthase subunit O, mitochondrial	P4HA1	Prolyl 4-hydroxylase subunit alpha-1

DNAJA3	DnaJ homolog subfamily A member 3, mitochondrial	SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1
KRT13	Keratin, type I cytoskeletal 13	DPM1	Dolichol-phosphate mannosyltransferase subunit 1
CKB	Creatine kinase B-type	SUCLG1	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial
DDX21	Nucleolar RNA helicase 2	UMPS	Uridine 5'-monophosphate synthase
ADCK3	Chaperone activity of bc1 complex-like, mitochondrial	LARS	Leucine--tRNA ligase, cytoplasmic
RPL19	60S ribosomal protein L19	ADCK3	Chaperone activity of bc1 complex-like, mitochondrial
P4HA1	Prolyl 4-hydroxylase subunit alpha-1	SLFN11	Schlafen family member 11
KRT73	Keratin, type II cytoskeletal 73	DNAJA3	DnaJ homolog subfamily A member 3, mitochondrial
RPLP1	60S acidic ribosomal protein P1	UQCRC2	Cytochrome b-c1 complex subunit 2, mitochondrial
ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	ALDH1B1	Aldehyde dehydrogenase X, mitochondrial
RPL7A	60S ribosomal protein L7a	ATP5O	ATP synthase subunit O, mitochondrial
ATP1A3	Sodium/potassium-transporting ATPase subunit alpha-3	PYCR2	Pyrroline-5-carboxylate reductase 2
MCM7	DNA replication licensing factor MCM7	RFC3	Replication factor C subunit 3
RFC3	Replication factor C subunit 3	GRPEL1	GrpE protein homolog 1, mitochondrial
RPL13A	60S ribosomal protein L13a	PYCR1	Pyrroline-5-carboxylate reductase 1, mitochondrial
RPL23A	60S ribosomal protein L23a	H2AFZ	Histone H2A.Z
KRT78	Keratin, type II cytoskeletal 78	MYH10	Myosin-10
POLDIP2	Polymerase delta-interacting	HSD17B10	3-hydroxyacyl-CoA dehydrogenase type-2

	protein 2		
SLC25A6	ADP/ATP translocase 3	RPL19	60S ribosomal protein L19
DNAJA1	DnaJ homolog subfamily A member 1	KRT27	Keratin, type I cytoskeletal 27
CORO1C	Coronin-1C	DCTN1	Dynactin subunit 1
HNRNPAB	Heterogeneous nuclear ribonucleoprotein A/B	ABCF1	ATP-binding cassette sub-family F member 1
NDUFA4	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	RPL13A	60S ribosomal protein L13a
SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	RPL7A	60S ribosomal protein L7a
RAN	GTP-binding nuclear protein Ran	HEXB	Beta-hexosaminidase subunit beta
SUCLG2	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial	DDB1	DNA damage-binding protein 1
SNRNP70	U1 small nuclear ribonucleoprotein 70 kDa	TUBG1	Tubulin gamma-1 chain
HIST1H3A	Histone H3.1	CBR4	Carbonyl reductase family member 4
YWHAQ	14-3-3 protein theta	SHMT2	Serine hydroxymethyltransferase, mitochondrial
PYCR2	Pyrroline-5-carboxylate reductase 2	EARS2	Probable glutamate--tRNA ligase, mitochondrial
DDX23	Probable ATP-dependent RNA helicase DDX23	DSP	Desmoplakin
CCAR1	Cell division cycle and apoptosis regulator protein 1	HAGH	Hydroxyacylglutathione hydrolase, mitochondrial
		DCD	Dermcidin
		PDK3	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 3, mitochondrial

	VPS4A	Vacuolar protein sorting-associated protein 4A
	SMC4	Structural maintenance of chromosomes protein 4
	SLC25A6	ADP/ATP translocase 3
	HNRNPAB	Heterogeneous nuclear ribonucleoprotein A/B
	DNAJA2	DnaJ homolog subfamily A member 2
	PYCRL	Pyrroline-5-carboxylate reductase 3
	INTS6	Integrator complex subunit 6

5.7A) and in percentage terms for both GST-PS1-Loop **(Figure 5.7B)** and GST-PS1-Loop FP/VS/AA **(Figure 5.7C)**. The GST-PS1-Loop dataset contained more proteins involved in both binding activities and structural activities; the GST-PS1-Loop FP/VS/AA dataset contained more proteins with a catalytic activity **(Figure 5.7A)**. These differences are also represented in the bar charts **(Figures 5.7B and 5.7C)** where the structural activity represents the biggest function in the GST-PS1-Loop dataset and catalytic activity is the biggest function in the GST-PS1-Loop FP/VS/AA dataset.

5.9 PANTHER Biological Processes of GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets

The PANTHER classification system was again used to investigate the biological processes involved in the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets. The PANTHER system again uses the GO terms for this classification system; as of 2011 the GO uses over 20000 different biological process terms when classifying proteins/genes [The Gene Ontology Consortium 2011]. The GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets are compared in absolute terms **(Figure 5.8A)**. The GST-PS1-Loop dataset is over-represented by the cellular component organisation and developmental processes and the GST-PS1-Loop FP/VS/AA dataset is overrepresented by the response to stimulus, metabolic and immune system processes. These over-representations can also be seen in relative terms by comparing the pie charts seen **(Figures 5.8B and 5.8C)**. The presence of two different actin proteins in the GST-PS1-Loop dataset explains the over-

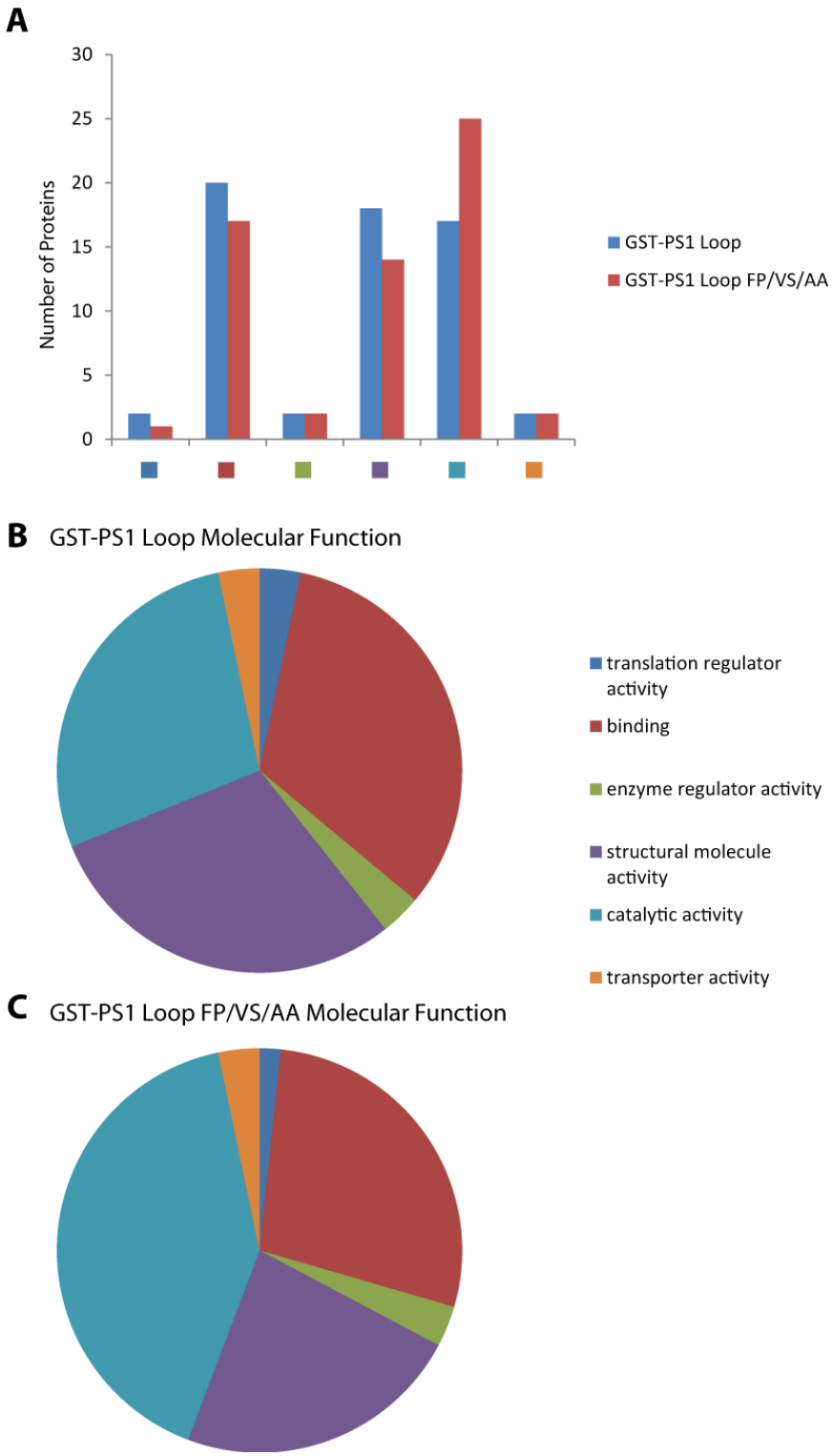


Figure 5.7 Molecular function analysis of GST-PS1-Loop and GST-PS1-Loop FP/VS/AA using PANTHER. The molecular functions of the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets were analysed using the PANTHER clsasification system. (A) The molecular function data was first compared in absolute terms in a bar chart. The percentage breakdown for the (B) GST-PS1-Loop and (C) GST-PS1-Loop FP/VS/AA datasets are also shown using pie charts. The key for the colour scheme used is shown on the right.

representation of the cellular component organisation and developmental processes in these dataset. Schlafen family member 11 (SLFN11) was a protein identified solely in the GST-PS1-Loop FP/VS/AA dataset, as can be seen in Table 5.4. This protein represented an important difference in terms of the response to stimulus and immune system processes that exist between the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets. Schlafen genes are upregulated in response to interferon and SLFN11 is a protein that has been shown to block HIV-1 replication inside host cells by selectively interacting with viral tRNA [Li *et al* 2012]. The other biological processes (cellular process, localisation, biological regulation, multicellular organismal and biological adhesion) were at similar levels to another across both datasets.

5.10 PANTHER Pathway Analysis of GST-PS1-Loop and GST-PS1-Loop FP/VS/AA Datasets

Following the analysis of the biological process involvement of the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets the PANTHER classification system was used to analyse the involvement of proteins within the different pathways. The GST-PS1-Loop dataset was found to be involved in 17 different pathways while the GST-PS1-Loop FP/VS/AA dataset was found to be involved in 21 different pathways **(Table 5.6)**. Fourteen pathways were represented in both datasets and for each dataset there were a number of unique pathways. As these unique pathways were each represented by a single protein they will be listed here. For the GST-PS1-Loop dataset there were 3 unique pathways represented: integrin signalling pathway (Actin, cytoplasmic 2), FGF signalling pathway (14-3-3 protein theta) and EGF

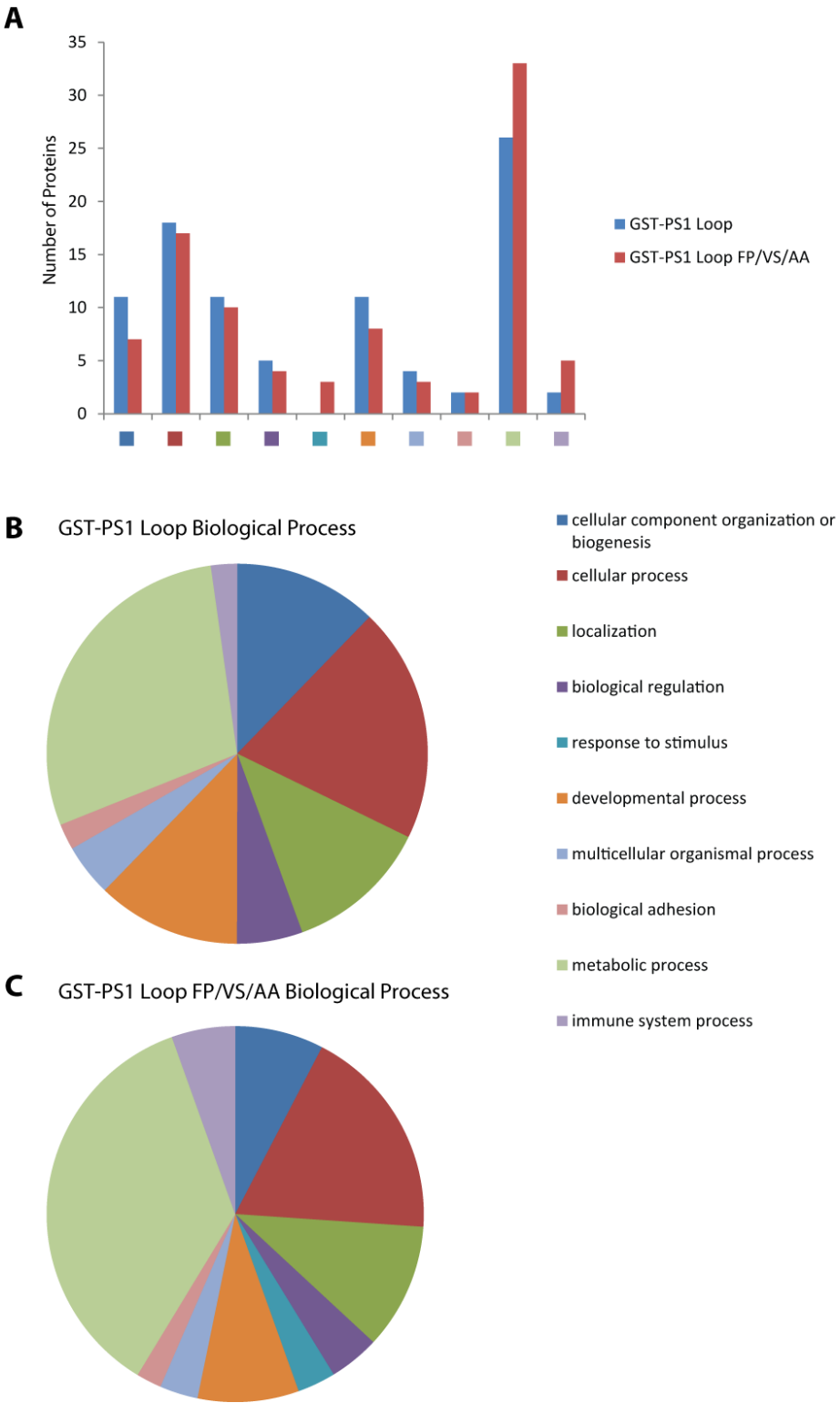


Figure 5.8 GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets analysed by biological processes using PANTHER. (A) The biological processes of the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets were examined using the PANTHER classification system and the resulting data was compared in absolute terms using a bar chart. The breakdown for biological processes were also shown in percentage terms for (B) the GST-PS1-Loop dataset or (C) GST-PS1-Loop dataset. The key for the colour scheme used is shown on the right.

receptor signalling pathways represented: (14-3-3 protein theta). For the GST-PS1-Loop FP/VS/AA dataset there were 7 unique pathways represented were ionotropic glutamate receptor pathway (Vesicle fusing ATPase/NSF), heme biosynthesis (Probable glutamate-tRNA ligase, mitochondrial), serine glycine biosynthesis (Serine hydroxymethyltransferase, mitochondrial), *de novo* pyrmidine riobonucleotide sysnthesis (Uridine 5'-monophosphate synthase), synaptic vesicle trafficking (Vesicle fusing ATPase/NSF), hedgehog signalling pathway (F-box/WD repeat-containing protein 11) and the Krebs cycle (Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial). Table 5.6 shows that the AD-associated presenilin pathway, the cytoskeletal regulation by Rho pathway and Inflammation-mediated by chemokine and cytokine signaling pathway showed a greater than 50% increase in number of pathway hits in the GST-PS1-Loop dataset as compared to the GST-PS1-Loop FP/VS/AA dataset. All other pathways showed a lesser than 50% difference in pathway hits when comparing pathways across the two datasets.

Table 5.6. List of pathways identified through PANTHER pathway analysis of the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets.

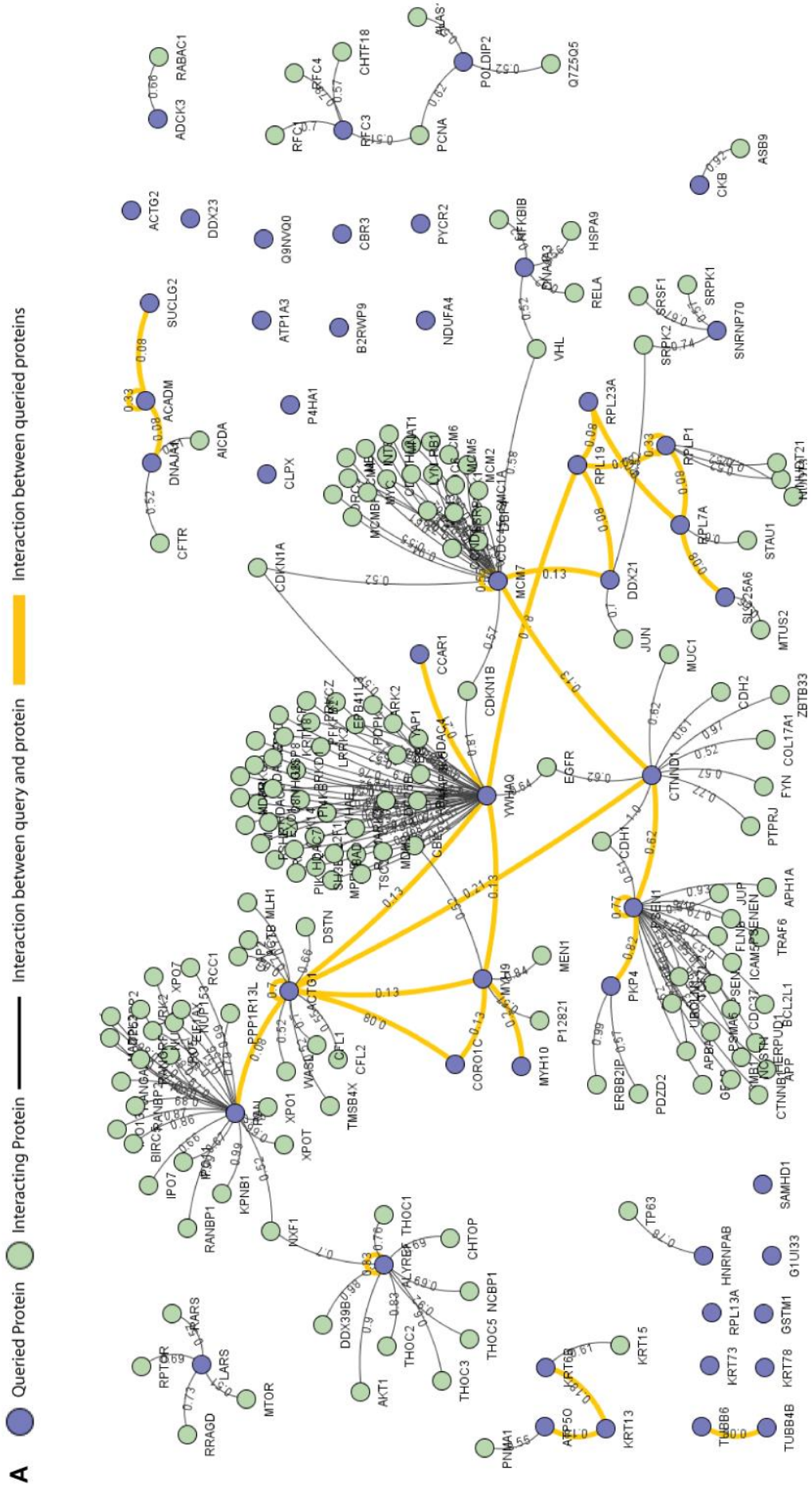
GST-PS1-Loop Dataset				GST-PS1-Loop FP/VS/AA Dataset			
Pathway	**# Genes**	**% of total genes**	**% of pathway hits**	**Pathway**	**# Genes**	**% of total genes**	**% of pathway hits**
Alzheimer disease-amyloid secretase pathway	1	1.80%	2.50%	Alzheimer disease-amyloid secretase pathway	1	1.80%	3.00%
Alzheimer disease-presenilin pathway	3	5.30%	7.50%	Alzheimer disease-presenilin pathway	1	1.80%	3.00%
Angiotensin II-stimulated signaling through G proteins and beta-arrestin	1	1.80%	2.50%	Angiotensin II-stimulated signaling through G proteins and beta-arrestin	1	1.80%	3.00%
Cadherin signaling pathway	3	5.30%	7.50%	Cadherin signaling pathway	1	1.80%	3.00%
Cytoskeletal regulation by Rho GTPase	6	10.50 %	15.00%	Cytoskeletal regulation by Rho GTPase	4	7.00%	12.10%
DNA replication	2	3.50%	5.00%	De novo pyrmidine ribonucleotides biosythesis	1	1.80%	3.00%
EGF receptor signaling pathway	1	1.80%	2.50%	DNA replication	1	1.80%	3.00%
FGF signaling pathway	1	1.80%	2.50%	Hedgehog signaling pathway	1	1.80%	3.00%
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	1	1.80%	2.50%	Heme biosynthesis	1	1.80%	3.00%
Huntington disease	5	8.80%	12.50%	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	1	1.80%	3.00%
Inflammation mediated by chemokine and cytokine signaling pathway	5	8.80%	12.50%	Huntington disease	4	7.00%	12.10%
Integrin signalling pathway	1	1.80%	2.50%	Inflammation mediated by chemokine and cytokine signaling pathway	3	5.30%	9.10%
Nicotinic acetylcholine receptor signaling pathway	4	7.00%	10.00%	Ionotropic glutamate receptor pathway	1	1.80%	3.00%
Notch signaling pathway	1	1.80%	2.50%	Nicotinic acetylcholine receptor signaling pathway	2	3.50%	6.10%
Parkinson disease	2	3.50%	5.00%	Notch signaling pathway	1	1.80%	3.00%
Proline biosynthesis	1	1.80%	2.50%	Parkinson disease	1	1.80%	3.00%
Wnt signaling pathway	2	3.50%	5.00%	Proline biosynthesis	3	5.30%	9.10%
				Serine glycine biosynthesis	1	1.80%	3.00%
				Synaptic_vesicle_trafficking	1	1.80%	3.00%
				TCA cycle	1	1.80%	3.00%
				Wnt signaling pathway	2	3.50%	6.10%

5.11 Mentha Interactome Mapping of GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets

The GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets contain lists of that were bound by the GST pulldown assay and identified through mass spectrometry The molecular functions, biological processes and cellular pathways of these

proteins have been characterised using the PANTHER classification system. Since these proteins are involved in multiple pathways and biological processes it was decided to look for pre-existing protein-protein interactions within the proteins found in these databases. By doing this it was hoped to reveal the presence of different protein-protein interaction nodes between the GST-PS1-Loop and the GST-PS1-Loop FP/VS/AA datasets, which could help reveal the function of the PS1 CUE domain. To do this the Mentha Interactome Browser was used (mentha.uniroma2.it/) [Calderone *et al* 2013], which contains protein interaction databases of published protein-protein interactions for 8 different species including humans. Each interaction is given a reliability score (between 0 and 1) that measures the reliability of the interaction based on both the interaction type and interaction method recorded in the published data. The gene names for both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA databases compiled in Table 5.6 were entered into the Mentha browser to provide a network map where each node is represented by a gene name and each link represents a known protein interaction. A reliability score of 0.5 was assigned for both datasets to provide a clear picture of these known protein-protein interactions. The GST-PS1-Loop dataset is shown in **Figure 5.9A** while the GST-PS1-Loop FP/VS/AA dataset is represented in **Figure 5.9B**. In both network maps queried genes are represented by purple nodes while green nodes represent genes that interact with queried genes with a reliability score of >0.5. Protein-protein interactions between nodes are represented by black lines while protein-protein interactions between queried genes are represented by yellow lines. PS1 itself, DNA replication licensing factor MCM7 and δ-catenin are present as major nodes in both datasets. RAN (GTP-binding nuclear protein Ran) and YWHAQ (14-3-3 protein θ) represent major nodes of protein-protein interaction that are unique to the GST-PS1-

Loop dataset. While the DDB1 (DNA damage-binding protein 1) and the E3 ligase FBXW11 (F-box/WD repeat-containing protein 11) represent the major nodes unique to the GST-PS1-Loop FP/VS/AA dataset. The GST-PS1-Loop FP/VS/AA dataset contains a greater number of known PS1 interacting proteins (plakophilin-4, desmoplakin, δ-catenin and Glutaryl-CoA dehydrogenase) than the GST-PS1-Loop dataset (plakophilin-4 and δ-catenin). In the GST-PS1-Loop dataset 16 proteins do not show protein-protein interactions with a reliability score of <0.5 while in the GST-PS1-Loop dataset 8 proteins have a reliability score of <0.5. These interaction maps **(Figure 5.9A/B)** demonstrate that PS1 was previously known to interact with two proteins (plakophilin-4 and δ-catenin) in the GST-PS1-Loop sample and four proteins [glutaryl-CoA dehydrogenase (GCDH), desmoplakin, plakophilin-4 and δ-catenin] in the GST-PS1-Loop FP/VS/AA dataset. As the interaction maps shows known interactions between the proteins present in both datasets. The interaction maps also show published interactions between identified proteins and other proteins. This can provide some links between different proteins identified in the datasets for example EGFR connects to both 14-3-3-θ and δ-catenin **(Figure 5.9A)** or β-catenin that connects to both PS1 and FBXW11 **(Figure 5.9B).** These protein-protein interactions may be able to provide some insight into the function of the PS1 CUE domain.


A
Queried Protein
Interacting Protein
Interaction between query and protein
Interaction between queried proteins

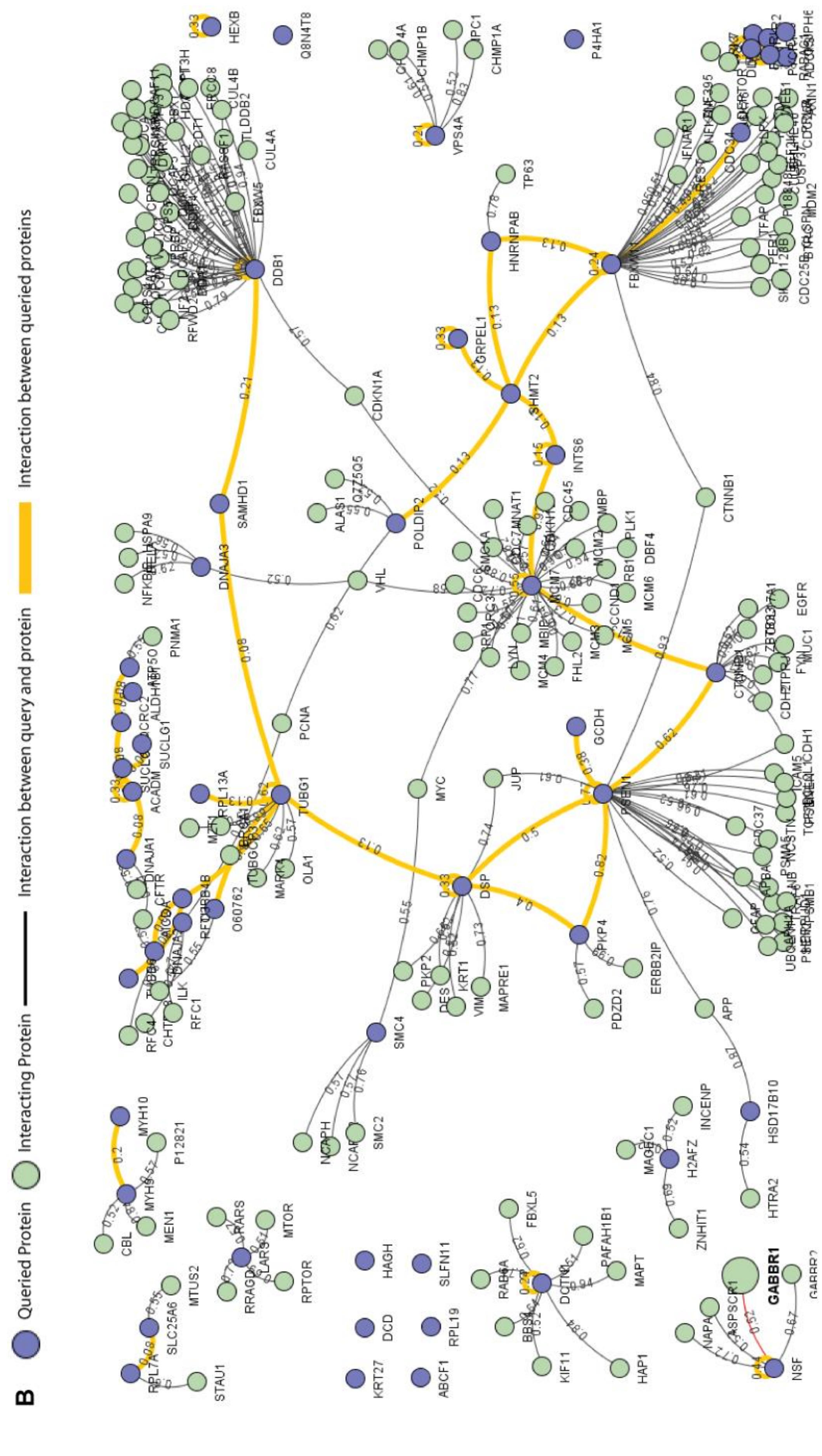

B
Queried Protein
Interacting Protein
Interaction between query and protein
Interaction between queried proteins

Figure 5.9 Mentha alignment of known protein-protein interactions of GST-PS1-Loop dataset. The proteins from the (A) GST-PS1-Loop dataset or the (B) GST-PS1-Loop FP/VS/AA dataset were analysed for protein-protein interactions using the Mentha search engine. A cut-off was applied such that any protein-protein with a reliability score under 0.50 was excluded from view. All proteins searched for are highlighted in purple and any protein-protein interactions between proteins searched for are coloured yellow. A colour scheme is included above both interactions maps.

Discussion

The aim of this chapter was to identify PS1 CUE domain binding proteins and to determine whether the PS1 CUE domain is involved in the intermolecular or intramolecular interactions of PS1. We hypothesised that the PS1 CUE domain was involved in regulating either intramolecular PS1:PS1 interactions or PS1 intermolecular protein-protein interactions. To test this hypothesis a GST-pulldown assay was devised, validated and used for a mass spectrometry-based proteomics assay. Using a recombinant PS1 loop domain that contains the CUE domain and a recombinant PS1 CUE domain mutant protein, we anticipated that it would be possible to distinguish between ubiquitinated proteins that bound to the PS1 intracellular loop domain and the PS1 CUE domain. Through the identification of PS1 CUE domain binding proteins we further hypothesised that it would be possible to determine whether the PS1 CUE domain was involved in γ-secretase-dependent or γ-secretase-independent PS1 function(s).

PS1 is known to form homo- and heterodimers, where dimers have been shown to form between the PS1 holoprotein, N-terminal and C-terminal fragments using a two-hybrid yeast assay [Hebert *et al* 2004]. In this study, no PS1 tryptic peptides corresponding to peptide sequences from outside the PS1 intracellular loop were identified in the mass spectrometry-based analysis **(Figure 5.6)**. This demonstrated that the intramolecular PS1:PS1 interaction is not regulated by either the intracellular loop or the CUE domain of PS1. This also strongly suggests that the function of the PS1 CUE domain is involved in regulating intermolecular PS1 protein-protein interactions.

In chapter 3 deletion of the PS1 CUE domain and mutation of conserved CUE domain residues were shown to be dispensable for the γ-secretase mediated cleavage of APP **(Figure 3.12)**, Notch **(Figure 3.14)** and IL-1R1 **(Figure 3.15)**. To further investigate whether the PS1 CUE domain was involved in γ-secretase function the proteins identified through mass spectrometry were analysed to look for other members of the γ-secretase complex, γ-secretase substrates or other type-1 transmembrane proteins. The γ-secretase protease complex is comprised of presenilins, Nicastrin [Yu et al 2000], Aph1 and Pen-2 [Hebert et al 2004]. None of these proteins were identified in either the GST-PS1-Loop or the GST-PS1-Loop FP/VS/AA samples which suggests that the PS1 CUE domain is not involved in the formation of the γ-secretase complex. In addition to the γ-secretase substrates that were examined here (APP, Notch and IL-1R1) there are over 100 known γ-secretase substrates [Haapasalo and Kovacs 2011]. No known γ-secretase substrates were identified through the mass spectrometry analysis of the GST-PS1-Loop or the GST-PS1-Loop FPVSAA samples. Only three type 1 transmembrane proteins were identified in either sample: the small integral membrane protein 4 (SMIM4), dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 (RPN1) and mitochondrial import inner membrane translocase subunit (TIM50). However, both RPN1 and TIM50 were also identified in the GST negative control sample so is most likely a result of non-specific binding. The SMIM4 is an uncharacterised protein of 70 residues in length that was discovered through the analysis of human cDNA [Mammalian Gene Collection (MGC) Program Team 2002]. These data further supports the conclusion that the PS1 CUE domain is not involved in γ-secretase cleavage and suggests a γ-secretase independent function for the PS1 CUE domain.

To investigate the involvement of the PS1 CUE in the intermolecular protein-protein interactions of PS1 the lists of mass spectrometry identified proteins from the GST, GST-PS1-Loop and GST-PS1-Loop FP/VS/AA samples were analysed and broken down to identify proteins that interacted with just the GST-PS1-Loop or the GST-PS1-Loop FP/VS/AA recombinant proteins. There 22 proteins that were shown to uniquely interact with the GST-PS1-Loop protein **(Table 5.3),** 31 proteins that uniquely interacted with the GST-PS1-Loop FP/VS/AA mutant **(Table 5.4)** and 26 proteins were identified in both samples **(Table 5.2)**. In the GST-PS1-Loop sample only δ-catenin and plakophilin-4 proteins have been previously shown to interact with PS1 [Tanahashi *et al* 1999; Stahl *et al* 1999] **(Figure 5.9A)**, while 4 proteins identified in the GST-PS1-Loop FP/VS/AA sample were previously known to interact with PS1 [δ-catenin, plakophilin-4, desmoplakin and Glutaryl-CoA dehydrogenase (GCDH)] [Raurell *et al* 2006; Soler-lopez *et al* 2011] **(Figure 5.9B)**. Desmoplakin was also identified in both the GST and GST-PS1-Loop samples but was present at a low level, below the applied cutoff.

The armadillo proteins plakophilin-4 and δ-catenin were two of the highest Mascot scoring proteins that were present in both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets. Both α-catenin and JUP were identified in the GST-PS1-Loop sample and β-catenin was identified in the GST-PS1-Loop FP/VS/AA sample. All of these proteins were identified through one peptide and so were below the cut-off level. With the exception of JUP, all of these armadillo proteins have previously been shown to interact with PS1 [Georgakopoulos *et al* 1999; Tanahashi *et al* 1999; Stahl *et al* 1999; Tesco *et al* 1998] **(Table 4.1)**. The armadillo proteins are involved in cell signalling, gene transcription and the cytoskeletal structure [Dusek and Attardi 2011]. The armadillo protein α-catenin can interact with both the E-

cadherin/β-catenin complex and with actin filaments where it acts to inhibit actin polymerisation [Drees *et al* 2005]. Overexpression of α-catenin leads to down regulation of Wnt signalling and developmental defects in *Xenopus laevis* [Sehgal *et al* 1997]. JUP, also known as γ-catenin, is involved in both desmosome and adherens junctions which can interact with the cytoskeleton [Dusek and Attardi 2011].

Adherens junctions are the transmembrane protein complexes that are involved in maintaining cell-cell contact and while desmosomes also form cell to cell linkages, they are also bound to the cells cytoskeleton [Dusek and Attardi 2011]. JUP is required during development but can also act as a negative regulator of β-catenin/Wnt signalling in zebrafish [Martin *et al* 2009]. Transport of JUP to desmosomes is controlled by 14-3-3-γ, where knockout of 14-3-3-γ leads to a decrease in desmosomes and adherens junctions between cells which can lead to male sterility in mice [Sehgel *et al* 2014]. Many non-small cell lung cancer cell lines show a reduction in JUP expression and restoration of JUP expression in these cells leads to a reduction in cancer cell migration [Sechler *et al* 2015].

Both δ-catenin and plakophilin-4 have previously been shown to interact with PS1 and are involved in cadherin signalling pathways [Tanahashi *et al* 1999; Stahl *et al* 1999]. Plakophilin-4 and δ-catenin are members of the plakophilin subcategory of armadillo proteins [Bass-Zubek *et al* 2009] and δ-catenin has previously been shown to interact with proteins involved in protein trafficking and the cytoskeleton [Koutras and Lévesque 2011]. δ-catenin knockout mice have deficits in synaptic plasticity [Israely *et al* 2004] and co-expression of PS1 with δ-catenin has been shown to reduce dendrite branching in HIH 3T3 cells [Kim *et al* 2006]. Plakophilin-4 and δ-catenin were the highest Mascot scoring proteins identified in both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA samples. However, as these proteins were

identified in both samples it demonstrates that these proteins interact with the PS1 intracellular loop domain rather than the PS1 CUE domain.

The BioGRID was used to investigate the ubiquitination status of the proteins that were only contained within the GST-PS1-Loop dataset **(Table 5.3)** to determine whether any of these proteins undergo K63-linked polyubiquitination. Ubiquitination of the histone H3 protein was previously demonstrated, though the specific type of ubiquitination was not determined [Karagianni *et al* 2008]. The published ubiquitination status of the remaining proteins had been determined through high throughput screening of ubiquitination sites so the type of ubiquitination was also not determined. 14-3-3-θ, which was identified solely in the GST-PS1-Loop sample **(Table 5.3)**, is known to interact with a large number of other proteins **(Figure 5.9A)**.

No 14-3-3 isoform has previously been shown to interact with the presenilins so the interaction between PS1 and 14-3-3-θ represents a novel interaction. 14-3-3-θ has been shown to undergo ubiquitination through a number of mass throughput ubiquitination screens [Danielsen *et al* 2011; Wagner *et al* 2011; Stes *et al* 2014]. These studies unfortunately do not determine the type of ubiquitination that occur at these sites so it is not currently known if 14-3-3-θ undergoes K63-linked polyubiquitination. 14-3-3 proteins can bind to phosphorylated serine and threonine residues and are involved in many cellular pathways including TLR signalling [Schuster *et al* 2011], apoptosis [Nomura *et al* 2003] and trafficking [Sehgal *et al* 2014]. 14-3-3 proteins are also able to bind to non-phosphorylated proteins [Obsil and Obsilova 2011]. There are seven 14-3-3 isoforms found in mammals (β, ε, η, γ, τ, ζ and θ) which show high sequence homology between the different isoforms [Obsil and Obsilova 2011]. While the 14-3-3 proteins have not previously been

shown to interact with the presenilins, the 14-3-3 proteins and the presenilins are known to be involved in some of the same cell signalling pathways such as apoptosis [Nomura *et al* 2003; Yagi *et al* 2008], trafficking [Nakamura *et al* 2010; Khandelwal *et al* 2007] and β-catenin/Wnt signalling [Nava *et al* 2014; Kawamura *et al* 1998].

14-3-3-θ has been shown to interact with proteins involved in TLR signalling such as IRAK4 and ILF2 [Schuster *et al* 2011]. A number of different 14-3-3 isoforms are known to be involved in the regulation of trafficking within the cell. 14-3-3-θ interacts with PKC-γ leading to the phosphorylation of the $GABA_{A\gamma2}$ receptor. This phosphorylation is required for the sorting of the $GABA_{A\gamma2}$ receptor to the cell membrane in Purkinje cells [Qian *et al* 2013]. The presenilins are involved in the regulation of apoptosis through both γ-secretase-dependent and -independent functions [Zeng *et al* 2015]. 14-3-3 proteins are also involved in the regulation of apoptosis through protein-protein interactions. The pro-apoptotic Bcl-2 protein Bax has been shown to interact with 14-3-3-θ and this interaction downregulates the incorporation of Bax into the mitochondria [Nomura *et al* 2003]. The presenilins also known to upregulate Bax mediated apoptosis via P53 and PSAP [Alves da Costa *et al* 2003; Zeng *et al* 2015].

The presenilins are known to interact with the β-catenin and other armadillo proteins [Levesque *et al* 1999] and are able to regulate β-catenin/Wnt signalling [Kawamura *et al* 1998; Zhang *et al* 2008]. The functions of these armadillo proteins are known to be regulated by their interaction with different 14-3-3 isoforms and 14-3-3-θ in particular is required for the dynein-dynactin mediated transport of N-cadherin/β-catenin complex from the ER [Nakamura *et al* 2010]. The presenilins have not previously been shown to interact with any 14-3-3 proteins but as has been described

here, the presenilins and 14-3-3 are involved in the same signalling pathways and are known to interact with similar proteins.

There were 31 identified proteins that solely interacted with the GST-PS1-Loop recombinant protein **(Table 5.4)**. These proteins bind to the intracellular loop domain of PS1 when the PS1 CUE domain is disrupted and represent proteins whose interaction with PS1 is downregulated by a functional PS1 CUE domain. One such protein, FBXW11, which is also known as β-transducin repeat protein 2 (βTrCP2), got the second highest Mascot score of the proteins identified only in the GST-PS1-Loop FP/VS/AA mutant dataset. Mammals have two different genes for βTrCP, BTRC and FBXW11 in humans, and the two βTrCP proteins are 88% similar in primary sequence [Nakagawa *et al* 2015]. The βTrCP proteins, which are both F-box containing proteins, can form part of the SKP1-CUL1-F-box (SCF) E3 ubiquitin ligase complex which targets substrates for proteasomal degradation [Kim *et al* 2015]. βTrCP itself is known to be ubiquitinated leading to its degradation by the UPS [Li *et al* 2004]. There are no published interactions between the βTrCP proteins and the presenilins. However the *C. elegans* presenilin homolog SEL-12 is known to interact with and be ubiquitinated by the F-box protein SEL-10 E3 ligase via the SCF complex [Li *et al* 2002]. Transcription of Fbxw7, the human homolog of SEL-10, is regulated by the presenilins and this genetic relationship in turn regulates EGFR stability [Rocher-Ros *et al* 2010]. Due to their high sequence similarity the βTrCP proteins do have some overlapping roles in promoting protein turnover and regulating cell signalling pathways, but βTrCP2 does also have some unique functions. While both βTrCP proteins may be found in the nucleus only βTrCP2 is observed within the cytoplasm [Lassot *et al* 2001]. The βTrCP proteins are known to interact with β-catenin leading to reduction in β-catenin levels and a reduction in β-

catenin induced gene transcription [Hart *et al* 1998]. Both βTrCP proteins upregulate NFκB signalling by promoting the ubiquitination and proteasomal degradation of IRAK1 and IκBα [Cui *et al* 2012; Suzuki *et al* 2000]. βTrCP2, but not βTrCP1, promotes the proteasomal degradation $p19^{ARF}$ to promote proliferation in MEFs [Nakagawa *et al* 2015]. βTrCP2 also regulates the proteasomal degradation of the prolactin-receptor [Li *et al* 2004]. Knockout of βTrCP2 was shown to be embryonically lethal in mice, whereas knockout of βTrCP1 has no major phenotypic effect [Nakagawa *et al* 2015; Nakayama *et al* 2003]. The differences in binding to 14-3-3-θ and βTrCP2 between the WT GST-PS1-Loop and the GST-PS1-Loop FP/VS/AA CUE domain mutant may represent an important difference if the PS1 CUE domain is involved in regulating cell signalling pathways.

A number of proteins found in both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets are involved in cellular transport and the cytoskeleton **(Figure 5.7 and Figure 5.8)**. The plakophilins are known to interact with Rho GTPase to regulate actin structures to affect cell morphology [Anastasiadis 2007], while the 2 actin proteins identified in the GST-PS1-Loop dataset (Actin, cytoplasmic 2 and Actin, gamma-enteric smooth muscle) are involved in the Rho cytoskeleton pathway **(Table 5.6)**. Myosin and tubulin proteins are present in the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets **(Figure 5.5)**. All of these data suggest a role for the PS1 intracellular loop domain in the regulation of the cytoskeleton. Plakophilin-4, δ-catenin and the tubulin proteins were identified in both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets, which could suggest that any role played by PS1 in cytoskeleton regulation is a function of the PS1 intracellular loop rather than the PS1 CUE domain. Proteins involved in protein trafficking were also identified in both datasets. The GTPase Ran was identified only in the GST-PS1-Loop dataset

and is an important node in terms of protein-protein interactions **(Figure 5.9A)**. Ran is known to undergo ubiquitination at K134, though the type of ubiquitination is unknown [Udeshi *et al* 2012]. Ran is involved in nuclear transport, microtubule formation and cellular mitosis [Sato and Toda 2010], and Ran is known to interact with the actin gene ACTG1 that was also identified in the GST-PS1-Loop sample **(Figure 5.9A)**. Ran is also known to be required for the translocation of β-catenin to the nucleus [Fagotto *et al* 1998]. The transcription factor TCF-4, which binds to β-catenin to co-regulate β-catenin induced gene transcription, also interacts with Ran and other nuclear pore complex proteins transport TCF-4 into the nucleus to upregulate TCF-4 gene transcription [Shitashige *et al* 2008]. The Vesicle-fusing ATPase, also known as NSF, which was identified only in the GST-PS1-Loop FP/VS/AA sample, is involved in vesicle trafficking within the cell where it interacts with SNARE proteins to promote membrane fusion [Zhao *et al* 2012]. NSF is involved in the transport of AMPA receptors to the post-synapse during long term potentiation in primary rat neurons [Yao *et al* 2008]. Expression of a dominant negative NSF mutant leads to a decrease in synaptic vesicle transport and alterations in actin structure in *Drosophila* neurons [Nunes *et al* 2006]. Expression of a truncated NSF mutant leads to alterations in synaptic formation in Zebrafish neurons and in a reduction in brain-derived neurotrophic factor from the synapse [Mo and Nicolson 2011]. Dynactin subunit 1, which was also only present in the GST-PS1-Loop FP/VS/AA sample, is part of the dynactin complex that is also known to be involved in microtubule related vesicle trafficking [Fu and Holzbaur 2014]. Dynactin regulates the sorting of phosphorylated Akt (p-Akt) to the microtubule network and knockdown of dynactin in cells leads to an increased susceptibility to apoptosis caused by a reduction in p-Akt levels and microtubule dysregulation [Jo *et al* 2014].

PS1 has been shown to play a γ-secretase independent role in protein and organelle trafficking within the cell [Khandelwal *et al* 2007; Naruse *et al* 1998; Lazarov *et al* 2007]. These data suggests that the intracellular loop of PS1 may play a role in regulating this function by interacting with the cytoskeleton and cytoskeleton interacting proteins. The GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets both contained proteins that are known to be involved in the regulation of cellular trafficking, some of which were common between both datasets [myosin 9 and 10, tubulin beta-4B and tubulin beta-6 **(Table 5.5)**]. Other proteins, such as Ran in the GST-PS1-Loop dataset and NSF or Dynatin subunit 1 in the GST-PS1-Loop FP/VS/AA dataset, were unique to a particular dataset. This suggests the PS1 CUE domain may play some role regulating PS1's role in protein and organelle trafficking. The PS1 CUE domain may help distinguish between different PS1 interacting proteins involved in different cellular trafficking pathways. The exact role played by the PS1 CUE domain in these processes remains to be fully deciphered.

Discussion

6.1 Overall Summary

The presenilins are best characterised as subunits of the γ-secretase protease complexes where they are involved in the regulated intramembrane proteolysis of over 100 different type 1 transmembrane proteins [Haapasalo *et al* 2012]. In addition to their role in the γ-secretase complexes the presenilins are involved in a number of γ-secretase-independent functions such as apoptosis [Zeng *et al* 2015], protein trafficking [Khandelwal *et al* 2007] and calcium homeostasis [Tu *et al* 2006]. In addition to the other members of the γ-secretase complexes, the presenilins are known to interact with over 100 other proteins that are involved in a variety of cellular processes and cell signalling pathways **(Table 4.1)** [McCarthy *et al* 2009; Soler-Lopez *et al* 2011].

The presenilins contain an intracellular loop domain between TMDs 6 and 7 that is the site of endoproteolysis [Oh and Turner 2005] and the site of some of the protein-protein interactions of the presenilins [Dumanchin *et al* 1999; Smith *et al* 2000]. Bioinformatic analysis of the PS1 intracellular loop sequence has shown the presence of GSK3β [Kirschenbaum *et al* 2001] and TRAF6 [Powell *et al* 2009] binding motifs. As a result of these interactions the presenilins are known to undergo GSK3β-mediated phosphorylation [Kirschenbaum *et al* 2001] and TRAF6-mediated K63-linked polyubiquitination [Yan *et al* 2013; Gudey *et al* 2014]. The TRAF6-mediated K63-linked polyubiquitination of PS1 leads to an increased stability of the PS1 holoprotein [Yan *et al* 2013]. Activity of the γ-secretase complexes has been shown to be modulated by the TRAF6-mediated K63-linked polyubiquitination of the γ-secretase substrates IL-1R1 [Elzinga *et al* 2009], $P75^{NTR}$ [Powell *et al* 2009]

and TGFβ Type 1 Receptor (TβR1) [Gudey *et al* 2014]. In addition to TRAF6 the presenilins are known to interact with and be ubiquitinated by the F-box containing protein SEL-10 in *C. elegans*, which regulates the degradation of the presenilin *C. elegans* homolog SEL-12 [Li *et al* 2002].

Further bioinformatics analysis of the primary sequence of the intracellular loops of the presenilins showed some similarity with an ubiquitin binding domain called coupling of ubiquitin to endoplasmic reticulum degradation (CUE) [Ponting 2000; Duggan *et al* 2015]. Ubiquitin molecules are bound to lysine residues and can be formed into polyubiquitin chains through any of the 7 lysine residues or the terminal glycine residue of ubiquitin [Komander and Rape 2012]. Ubiquitin binding domains can regulate protein function by binding to ubiquitin in either mono- or polyubiquitinated form and transmit the ubiquitination signal to regulate downstream cellular signalling [Chen and Sun 2009]. CUE domains are typically 40-45 residues in length and form a three helix secondary structure [Hurley et al 2006]. CUE domains contain a highly conserved phenylalanine-proline (FP) and less well conserved di-leucine motifs [Ponting 2000] and have been shown to bind to both monoubiquitin [Shih *et al* 2003] and polyubiquitin [Donaldson *et al* 2003; Liu *et al* 2014]. CUE domains have been shown to be involved in regulating ubiquitination [Liu *et al* 2014], autophagy [Lu *et al* 2014], protein turnover [Rego *et al* 2012] and cell signalling [Kishida *et al* 2005].

In this study the presenilins are shown to contain putative ubiquitin binding CUE domains in the intracellular loops between TMDs 6 and 7 (residues 270-314 in PS1 and 277-320 in PS2). This putative PS1 CUE domain contains the highly conserved FP motif at residues F283/P284 and in place of the di-leucine motif it has the V309/S310 motif. Using an ubiquitin binding assay PS1 and PS2 are shown to bind

preferentially to K63-linked polyubiquitin over K48-linked polyubiquitin; deletion of the CUE domains of the presenilins abolishes the ubiquitin binding functions of these proteins **(Figure 3.3 and Figure 3.5)**. Mutation of V309/S310 motif, but not the F283/P284 motif, was shown to abolish PS1 CUE domain function using both *in vivo* and *in vitro* ubiquitin binding assays **(Figure 3.4 and Figure 3.10)**. Next the PS1 CUE domain was shown to be dispensable for PS1 endoproteolysis in PS1 knockout MEF cells **(Figure 3.11)**. Deletion of the PS1 CUE domain or mutation of the conserved F283/P284 and V309/S310 motifs was then shown to have no effect on the γ-secretase mediated cleavage of APP **(Figure 3.12 and Figure 3.13)**, Notch **(Figure 3.14)** and IL-1R1 **(Figure 3.15)**. These data demonstrated that the PS1 CUE domain was not involved in γ-secretase activity and suggested a γ-secretase independent role for the PS1 CUE domain. We hypothesised that the PS1 CUE domain was involved in regulating PS1 intermolecular protein-protein interactions or regulating intramolecular PS1:PS1 interactions.

To investigate the function of PS1 CUE domain a list of PS1 interacting proteins and their published ubiquitination status was compiled **(Table 4.1)** and then restricted to PS1 interacting protein known to undergo K63-linked polyubiquitination **(Table 4.2)**. $P75^{NTR}$ and IL1-R1, which are known to undergo K63-linked polyubiquitination, were not shown to interact with the PS1 holoprotein or the PS1 ΔCUE mutant **(Figure 4.1 and Figure 4.2)**. The PS1 CUE domain was shown to be dispensable for the interaction between PS1 and TRAF2 **(Figure 4.8)** and RIP1 **(Figure 4.9)**. The E3 function of TRAF6 was shown to be required for the interaction between PS1 and TRAF6, while deletion of the PS1 CUE domain had no effect on this interaction **(Figure 4.3)**. While deletion of the PS1 CUE domain had no effect on the PS1-TRAF6 interaction, mutation of both the conserved F283/P284

and V309/S310 motifs in tandem reduced this interaction **(Figure 4.4)** but did not affect the TRAF6 mediated increased stability of the PS1 holoprotein **(Figure 4.5)**.

To further investigate the function of the PS1 CUE domain a mass spectrometry-based proteomics approach was used. The PS1 CUE domain containing GST-PS1-Loop recombinant protein or the GST-PS1-Loop FP/VS/AA CUE domain mutant were incubated with lysate from HEK293T cells and PS1 interacting proteins were purified using affinity chromatography **(Figure 5.5)** and then identified using mass spectrometry. Proteins that bound to both the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA were compiled into **Table 5.2** while proteins that bound to either GST-PS1-Loop or GST-PS1-Loop FP/VS/AA alone were compiled **(Table 5.3 and Table 5.4)**, respectively. Bioinformatic tools were then used to decipher shared and unique protein functions and protein-protein interactions that existed between the proteins found in the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets. Analysis of the proteins identified in the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA demonstrated that these proteins are involved in a variety of cellular processes including gene transcription, cellular trafficking, cytoskeleton organisation and the ubiquitin proteasome system. The armadillo proteins plakophilin-4 and δ-catenin were identified in both the GST-PS1-Loop **(**and GST-PS1-Loop FP/VS/AA datasets **(Table 5.2)**, while the armadillo proteins JUP and α-catenin were found solely in the GST-PS1-Loop dataset and β-catenin was found solely in the GST-PS1-Loop FP/VS/AA dataset. PS1 is known to localise at the plasma membrane where the γ-secretase mediated cleavage of E-cadherin is known to regulate the breakdown of adherens junctions [Marambaud *et al* 2002]. No cadherin protein was identified in the mass spectrometry analysis of either the GST-PS1-Loop or the GST-PS1-Loop FP/VS/AA samples therefore the interaction between these cytoplasmic desmosome

proteins and PS1 may occur away from the membrane. This could suggest that PS1 may play some additional role in regulating the function of these proteins.

A greater number of proteins were identified solely in the GST-PS1-Loop FP/VS/AA dataset (31 proteins) than in the GST-PS1-Loop dataset (22 proteins). This may suggest that the PS1 CUE domain may play a role in downregulating a number of PS1's protein-protein interactions. In other words the presence of a functional K63-linked polyubiquitin binding CUE domain may prevent PS1 interacting with a number of other proteins such as the E3 ligase βTRCP2, SLN11, the transport protein NSF etc. **(Table 5.4).** Cytoskeletal proteins myosin and tubulin proteins were identified in both mass spectrometry datasets **(Table 5.2)**, while two actin proteins were identified solely in the GST-PS1-Loop dataset **(Table 5.3).** NSF, also known as vesicle-fusing ATPase, and dynactin subunit 1 were identified solely in the GST-PS1-Loop FP/VS/AA dataset **(Table 5.4)**. NSF is involved in vesicle transport and synaptic structure [Nunes *et al* 2006; Mo and Nicolson 2011] and dynactin subunit 1 is involved in vesicle trafficking via the microtubule network [Fu and Holzbaur 2014]. The GTPase Ran was identified solely in the GST-PS1-Loop dataset **(Table 5.3)** and is also involved in the regulation of nuclear transport and microtubule assembly [Sato and Toda 2012]. These proteins **(Table 6.1)** are involved in cellular processes such as cell signalling and transport that PS1 is known to be involved in and so are strong targets for future investigation.

6.2 Future Perspectives

To investigate the function of the PS1 CUE domain a list of PS1 interacting proteins known to undergo K63-linked polyubiquitination was compiled **(Table 4.2)**. The necessity of the PS1 CUE domain for regulating these interactions was examined for

Table 6.1 PS1 interacting proteins identified through mass spectrometry for future investigation.

Dataset	Protein	Function
GST-PS1-Loop	δ-catenin	Cytoskeleton/signalling
	Plakophilin-4	Cytoskeleton/signalling
	Ran	Trafficking
	14-3-3-θ	Trafficking
GST-PS1-Loop FP/VS/AA	δ-catenin	Cytoskeleton/signalling
	Plakophilin-4	Cytoskeleton/signalling
	βTRCP2	Ubiquitination
	NSF	Trafficking
	SLFN11	Translation

three proteins only (TRAF2, TRAF6 and RIP1). All of these proteins were shown to interact with PS1 by previous members of the lab [Powell unpublished, Powell *et al* 2009, Harte unpublished]. As previously discussed in the discussion section of chapter 4 the aspartyl protease BACE1, the AICD interacting protein Fe65 and the AD associated protein Tau should all be looked at as a future target for studying PS1 CUE domain function as these proteins are associated with regulated intramembrane proteolysis/γ-secretase activity or symptomatic of AD. While the data presented here strongly suggests that the PS1 CUE domain is not involved in the regulation of γ-secretase activity, investigating the interaction of these proteins and PS1 could further demonstrate that this domain is involved in a γ-secretase independent PS1 function. Alternatively this could demonstrate a role for the PS1 CUE domain in the regulation of BACE1 mediated ectodomaining shedding [Hebert *et al* 2003], AICD/Fe65 mediated gene transcription [Vazquez *et al* 2008] or the turnover of ubiquitinated Tau [Babu *et al* 2008].

This research utilised a mass spectrometry based proteomics based approach to identify PS1 CUE domain interacting proteins to help decipher the function of the

PS1 CUE domain. From this approach 74 novel PS1 interacting proteins were identified. However, this approach has not demonstrated a clear function for the PS1 CUE domain but has provided a number of targets for future investigation **(Table 6.1)**. Firstly, the proteomics approach used unstimulated HEK293T cells to identify PS1 CUE domain interacting proteins. This approach may have hidden any interactions that may be dependent on cells being stimulated by external factors such as cytokines or any interactions that may be particular to certain cell types. Since this proteomics based approach did not fully elucidate the function of the PS1 CUE domain alternative approaches could in future be used to investigate PS1 CUE domain function. During this research attempts were made to produce a MEF PSDKO cell line that stably expressed the PS1 F283A/P284A/V309A/S310A mutant but unfortunately a functional cell line was not produced. This cell line was going to be used to investigate whether the PS1 CUE domain was involved in a number of PS1 γ-secretase independent functions such as Ca^{2+} homeostasis [Tu *et al* 2006] or protein or organelle trafficking [Naruse *et al* 1998, Khandelwal *et al* 2007]. This cell line could also be used to determine whether the PS1 F283A/P284A/V309A/S310A mutant itself suffered from any disruptions in terms of localisation within the cell as compared to wild type PS1. The creation of a stably expressing PS1 F283A/P284A/V309A/S310A cell line could be of great use in deciphering PS1 CUE domain function in the future.

The armadillo proteins can be found at adherens junctions and desmosomes where they interact with transmembrane adhesion proteins like the cadherins [Dusek and Attardi 2011]. In addition to that β-catenin, JUP and plakophilin-4 are able to translocate to the nucleus to regulate transcription in conjunction with the transcription factor TCF-4 [Dusek and Attardi 2011]. As the intracellular loop

domain of PS1 was shown here to interact with desmosome proteins such as δ-catenin, plakophilin-4, desmoplakin and JUP the co-localisation of PS1 with desmosomes should be a target for future investigation. No cadherin proteins were identified in the mass spectrometry analysis of either sample suggesting that the interaction of PS1 and these proteins could also be involved in other armadillo protein function. Presenilin is also involved in the regulation of β-catenin/TCF-4 mediated gene transcription [Raurell *et al* 2008] as are plakophilin-4 and δ-catenin [Dusek and Attardi 2011]. Previously examined CUE domains are known to regulate the transduction of cell signalling pathways such as the TAB2 CUE domain which is known to be essential for induction of NFκB signalling by promoting the interaction of TRAF6 and IKKα [Kishida *et al* 2005]. The role of the PS1 CUE domain in the regulation of β-catenin/TCF-4 gene mediated transcription was not investigated here and should provide another route for determining the function of PS1 CUE domain.

PS1 is known to play a role in regulating the transport of β-catenin [Noll *et al* 2000; Meredith *et al* 2002]. The activity and transportation of the armadillo proteins has also been shown to be regulated by other proteins which were identified in the GST-PS1-Loop and GST-PS1-Loop FP/VS/AA datasets, however, the 14-3-3-θ isoform, which was identified only in the GST-PS1-Loop dataset, has not previously been shown to interact with any of the armadillo proteins identified here. 14-3-3 proteins have been shown to be involved in the transport of JUP to desmosomes [Sehgal *et al* 2014] and β-catenin into the nucleus [Nava *et al* 2014]. Disruption of the 14-3-3-γ mediated transport of JUP leads to a reduction in desmosome formation and knockout of 14-3-3-γ causes sterility in male mice [Sehgal *et al* 2014]. The involvement of 14-3-3 proteins in the transport of these armadillo proteins could represent a future target for investigating the function of the PS1 CUE domain. As

other 14-3-3 isoforms are known to interact with other armadillo proteins to regulate their transport determining whether 14-3-3-θ interacts with any of these armadillo proteins should be investigated. If this 14-3-3 isoform is shown to interact with desmosome complex proteins it could begin to elucidate a function for PS1 and 14-3-3-θ in the regulation of desmosome assembly. Additionally, 14-3-3-θ is known to be ubiquitinated within the cell though the type of ubiquitination is not known [Danielsen *et al* 2011; Wagner *et al* 2011; Stes *et al* 2014]. It should be determined whether 14-3-3-θ is a target of K63-linked polyubiquitination and test whether the interaction between PS1 and 14-3-3-θ requires the K63-linked polyubiquitination of 14-3-3-θ.

βTRCP2, which was identified only in the GST-PS1-Loop FP/VS/AA dataset, is an E3 ligase that forms part of the SCF complex that mediates the proteasomal degradation of proteins [Kim *et al* 2015]. As PS1 is known to be ubiquitinated by the F-box protein SEL-10 via the SCF complex in *C. elegans* [Li *et al* 2002] it should be investigated whether PS1 is a target for βTRCP2 mediated ubiquitination and proteasomal degradation. CUE domains are known to be involved in the regulation of polyubiquitin chain formation. The CUE domain of Vps9 is known to be required for the mono-ubiquitination of Vps9 [Shih *et al* 2003]. Additionally, since βTRCP2 only interacted with the GST-PS1-Loop FP/VS/AA CUE domain mutant the effect of deletion or mutation of the PS1 CUE domain on βTRCP2 activity could be investigated. βTRCP2 is known to regulate the proteasomal degradation of α-catenin and β-catenin [Kim *et al* 2015] to downregulate the transcriptional activity of β-catenin [Fuchs *et al* 1999]. As βTRCP2 and PS1 can have opposing effects on the activity of the armadillo proteins they represent another potential source for future investigation into the function of the PS1 CUE domain. Schlafen-11 (SLFN11) was

identified only in the GST-PS1-Loop FP/VS/AA dataset **(Table 5.4)**. The function of SLFN11 is not well characterised at present but it is known to block HIV-1 replication within the cell by interacting with viral tRNA [Li *et al* 2012]. Mass spectrometry analysis of Notch ICD (NICD) interacting proteins purified from nuclear fractions showed that SLFN11 interacts with the NICD [Yatim *et al* 2013]. The PS1 interacting protein FBXW7 was also identified in this study and is known to regulate Notch activity with PS1 [Li *et al* 2002; Rocher-Ros *et al* 2010]. The interaction between PS1 and SLFN11 might provide a novel means through which PS1 can modulate Notch activity and should be looked into further.

PS1 is known to be involved in the regulation of protein and organelle trafficking and cytoskeleton organisation [Gandy *et al* 2007; Khandelwal *et al* 2007]. PS1 is also known to regulate axonal transport through modulating the activity of the kinesin-1 transport protein [Lazarov *et al* 2007] and is known to regulate the structure of the cytoskeletal protein filamin [Lu *et al* 2010]. The transport protein Ran is known to be ubiquitinated; high throughput ubiquitination screening has only provided a site of ubiquitination and not shown the ubiquitination type [Udeshi *et al* 2012]. Discovering whether Ran is a target for K63-linked polyubiquitination should be a target for future investigation before determining whether Ran ubiquitination is required for its interaction with PS1. As these transport proteins were identified in different datasets it may begin to suggest a role for the PS1 CUE domain in switching between different cellular transport pathways. PS1 is involved in transport of both proteins [Naruse *et al* 1998] and organelles [Khandelwal *et al* 2007] and in the regulation of axonal transport [Dolma *et al* 2014]. Therefore the affect of PS1 CUE domain deletion or mutation in the regulation of protein transport within cell would be a target for future investigation. As NSF regulates vesicle transport to

synapses [Nunes *et al* 2006] and Ran regulates nuclear transport [Sato and Toda 2012] differences in the localisation of wild type PS1 compared to PS1 CUE domain mutants within cells may demonstrate the function of the PS1 CUE domain. If the PS1 CUE domain mutants show an increase in vesicle co-localisation compared to wild type PS1 it would demonstrate that the PS1 CUE domain is involved in controlling PS1 localisation via its interaction with NSF within the cell. The CUE domain of Vps9 is required for the localisation of Vps9 to endosomes [Shideler *et al* 2015] and the CUE domain of Tollip acts in a similar manner, where binding of ubiquitin to its CUE domain causes Tollip to detach from membranes [Mitra *et al* 2013]. Mutation of the gp78 CUE domain is known to increase mobility of gp78 within the ER [St-Pierre *et al* 2011]. Therefore, both other known CUE domains and PS1 itself are known to be involved in the regulation of cellular trafficking and protein localisation. Whether the PS1 CUE domain plays a role in cellular trafficking remains to be determined and should be a major avenue for future investigation of PS1 CUE domain function.

In conclusion, the presenilins are shown here to contain K63-linked polyubiquitin chain binding CUE domains. The function of the PS1 CUE domain is shown to be dispensable for γ-secretase activity or interaction with γ-secretase substrates known to undergo K63-linked polyubiquitination. This suggests that the function of the PS1 CUE domain is involved in a γ-secretase independent role of PS1. The data presented here does not confirm the function of the PS1 CUE domain but the identification here of previously known and novel PS1 interacting proteins provides a number of avenue for future research into PS1 CUE domain function and the role of ubiquitin in regulating PS1 activity.

Appendices

Table 7.1 Total list of proteins identified in the mass spectrometry of the GST sample

P16152	P07910	Q13283	Q9P035	P51610	Q7L014	O43324	Q9H3N1	O94925	Q9BZZ5
P21266	P21796	Q96KK5	Q06830	P35249	P12268	Q96DI7	Q13416	Q86UK7	O14681
P04264	Q9UQE7	Q04695	O94874	Q02978	O95347	P40429	P63010	Q8N5C8	Q8NE71
P09211	P31943	Q96CX2	P11177	O14929	O14979	O00429	P55084	P05109	Q15287
P13645	P11940	P42167	Q14739	Q6Y7W6	P62263	Q15758	P49643	P05386	Q8N0X7
P28161	O00571	P45880	Q96AG4	P16989	P40926	Q16181	P23919	P50395	Q92544
P35527	P13639	P17812	P26373	P05198	P61088	Q13011	P62873	Q9BTT0	O00303
P10809	P06899	P12270	P78371	P0CG48	P17987	P08865	Q6NXT2	P35250	P31350
P35908	P49768	Q7KZF4	P00338	Q8N8S7	P24539	O96013	P22102	Q96QK1	P60228
P08107	P06733	P46783	O14880	Q15269	Q9BXP5	O75390	Q2TAY7	P62249	Q9UKM9
P07437	P06748	P62937	P27816	P14678	Q02543	Q14566	P49207	Q96C36	P24666
P08670	O60814	P30050	P16615	P56192	Q8WU90	P23284	Q06203	Q8TCT9	O00746
P22626	P19338	Q8NC51	P32969	P12277	Q9NVI7	P23258	Q14165	Q9BW92	P54886
P60709	Q07065	O75533	Q01081	Q9BPW8	P09622	P83731	Q16720	Q6UXN9	Q9NUU7
P52272	Q15029	P02768	O75489	Q14152	O75534	P36578	P62857	Q9BY77	P33991
P68104	P78347	P08559	Q86VP6	O94906	P27348	P15311	P46777	O60762	Q9UHI6
P09651	Q9NZI8	Q9Y230	P18085	P62280	P48643	Q9BSD7	O00231	P15924	P81605
P09874	P06576	Q15046	O60506	P22234	O95573	P50454	Q9BYE9	P46781	Q9BU23
Q9BVA1	Q13151	P62913	Q00325	P50914	Q14980	Q9Y4P3	Q15785	Q9NRZ9	Q8WUM4
P04350	P04843	Q9BWF3	P36873	P09661	Q9NSD9	P53999	P15927	Q96CW5	Q9BYJ9
Q08211	Q13310	Q9UQ80	P28331	P35637	Q8N1F7	Q13155	Q99497	Q92878	P39748
Q14204	P42704	P29692	P55884	P62899	Q99613	Q9UMS4	P01708	Q92997	Q15363
P68363	P62829	P12004	P27708	P61513	O00461	O95782	P62826	Q14684	O60645
Q9BQE3	Q04637	Q13813	Q13765	P62906	P61026	P40227	P61254	Q12788	Q16186
P08238	P62701	P47897	P62277	O00425	P06493	Q9UNL2	P62266	P22314	P56556
O15217	P52597	P07195	P33176	P62140	O95831	Q8TCJ2	Q12874	Q13242	P62714
P35232	P12956	P50991	P62269	Q5BKZ1	Q32MZ4	O43242	Q06265	Q6P1J9	Q10471
P07900	Q15393	Q16891	P49368	Q07021	Q99729	Q9P2J5	P57678	O00483	Q8WWY3
Q00839	P46782	P18124	Q99459	Q99798	P46977	P55209	Q9BVK6	P55265	P07237
Q05639	P39656	Q15717	P55786	P31153	P56134	P35998	O43684	P08621	Q9BXT5
P13647	P31942	P43243	P62241	P49755	Q07955	Q9Y490	P84098	Q86TJ2	Q9HB71
P26641	P39019	P23246	P62081	P36542	P62195	Q09028	P05387	Q9BTV4	Q7Z2T5
P41252	Q9Y4U1	P46940	P0CW22	P27694	Q04837	P40616	P13674	O00139	P18077
P02538	Q92945	Q92499	Q92688	Q96PK6	Q99832	P08579	A8MWD9	Q09161	P04075
Q96S19	P04844	P24534	P51148	Q9Y262	Q92900	O95202	P46087	P54709	P84090
Q00610	O43390	P05141	Q8N1G4	Q9UJS0	P46779	Q6UWP7	P51531	Q6PJT7	P48735
P60842	Q15084	P27635	P55060	Q13200	Q13868	Q9H9B4	O75947	P12814	Q9H672
P07814	Q86YZ3	Q9Y265	P13010	Q14683	Q9UHB9	Q99615	Q9UBU9	Q14444	P17980
Q99623	P52292	O43143	P61353	Q14318	P46778	P48047	Q8N766	P22570	Q8N163
P26599	P38919	P11021	Q7RTS7	Q00341	Q9BTC8	P05455	Q16630	P40938	P30419
P61978	Q9Y3I0	P63244	Q9Y285	Q9H0D6	Q02790	P62750	O95292	Q5HYI8	Q9H0S4
O75643	P51991	Q9BUJ2	Q15366	Q96S52	Q15645	Q9Y295	O94905	P51812	Q9UJZ1
Q12906	P54136	O43813	O75396	P51114	P26368	P23921	P46776	P29374	Q9NVI1
P11586	P78527	Q3ZCQ8	Q01813	Q9Y3B4	Q9BSJ8	Q8WY22	P30876	Q9P0I2	P35606
P51659	P14625	P67809	Q03252	P62888	P42677	Q92643	Q9Y3E0	Q8IYB3	
Q13263	P14866	P26196	P04181	Q13148	Q9NTJ3	P50402	Q9BUQ8	O14980	
P49411	P50990	P61247	P00918	P25205	P62244	P18754	Q9NQ29	Q9UQ16	
P25705	P23396	P23528	Q15019	P53621	P33992	P20042	Q9NPD3	O95168	
P68032	P05023	P84085	Q13347	P15880	Q7Z2W4	P37837	Q9BVP2	Q9NTZ6	
Q12931	P37802	P38646	Q14974	Q9NX63	O43809	Q07666	Q9P2B4	Q15006	
P02533	P39023	P41091	Q13573	Q9Y237	P60866	O75306	Q8NFW8	Q96HS1	
P20700	O43175	P39687	P46060	P62851	Q9Y5A9	P35268	P08243	P33993	
P14868	Q01082	P00403	Q9UHX1	Q14203	P62805	O75821	O75369	P13804	
P08779	P51572	P63173	P37108	O95793	P42285	P62847	Q96KR1	P34897	
Q92841	P49327	P00367	P62753	Q96EP5	P35580	P62306	Q02809	Q96FZ2	
P05388	P38159	P52209	Q5D862	Q7L2E3	Q00796	Q9NZT1	Q9Y6M9	P02545	
P04406	Q14103	Q15233	P32119	P43897	Q52LJ0	Q15459	Q6YN16	Q8IX01	
Q6P2Q9	O00148	P62495	O95232	P51570	P36507	Q9BZJ0	Q9BV38	P33240	
P17844	P61204	P40222	Q9UN86	Q9Y3A5	P62318	P51149	O96000	Q12824	
Q12905	P11142	Q13409	Q9Y224	Q96I24	P50213	O95104	P53396	Q01105	

Table 7.2 Total list of proteins identified in the mass spectrometry of the GST-PS1-Loop sample

P49768	Q9BUF5	P63267	Q15393	P40938	P07195	P05109	Q8IX12	P23284	P61313
P16152	Q99623	O43175	Q3ZCQ8	P40429	Q99613	Q13724	Q9Y4P3	Q9UQE7	P02768
P21266	Q96S19	P62269	Q3ZCQ8	Q92688	O75533	Q9HAV7	Q16531	Q9NTJ3	Q13547
P09211	P11142	Q9NZI8	Q14204	Q15233	P06576	Q9UJS0	Q16795	Q9BYJ9	P46779
O60716	O15217	Q6P2Q9	Q8NI60	Q07021	Q9Y3Z3	P56182	P78527	Q9P258	Q6I9Y2
P04264	P06748	P18124	P84098	O94906	P62826	P56182	P12268	Q9NSD9	P04075
P35527	P61978	O75643	P13674	P62750	Q96I99	Q9BRX2	O60762	P55081	Q96HL8
P13645	P07814	P12956	P16989	Q8N1N4	O60506	Q8IWZ3	P62888	P51570	Q5T2N8
P28161	P17844	Q15029	P63173	Q9Y2S7	Q9UMS4	Q8WY22	Q9NZ01	P61026	Q7L2E3
P35908	P07910	O76031	P35637	Q14203	Q02543	Q01082	P24390	O43684	P30419
P08107	P19338	P23246	Q9P035	Q96KR1	P08621	Q8N8S7	P11172	P60660	P17980
P68371	P62829	O43390	P26599	P17987	Q9UBB4	P62249	P15924	P46977	P62280
P07437	P41252	Q15717	P23396	Q9Y5M8	P50914	Q9BUQ8	Q15366	Q9Y5A9	P84090
P52272	Q12905	Q9P2J5	Q86Y46	Q03252	P11177	Q92499	Q9BVP2	Q96DI7	Q15019
O75828	P14868	Q12906	P05141	P62277	P04637	P0C0S5	P46087	Q96HP4	P35250
Q9BVA1	P35232	P47897	P12004	P12236	P10619	P18754	P0CG48	Q6YN16	Q13310
P26641	Q9Y4U1	P35580	P83731	P31689	Q8WUK0	Q14839	Q13813	P81605	O76061
P68104	P35579	P35580	Q02878	Q32MZ4	P08579	P39019	P27816	P07237	
P68363	P24534	Q00610	Q9BUJ2	P62851	P26640	Q9H0D6	Q13045	P62917	
P60709	Q9BSD7	P48047	P46778	P62851	P62195	P22087	Q13155	Q01469	
P63261	O43813	P31943	P05386	Q9Y285	P25205	P26196	Q7KZF4	Q16775	
P09488	P67809	Q9Y230	O43143	Q9Y285	P68431	P46781	Q9UNX3	P26368	
P02533	P51659	Q02978	P11310	Q9ULV4	P08559	P60866	Q09028	P54136	
P49411	P11021	Q96EY1	Q9UHX1	P60842	Q9BQ67	Q9NQT5	Q96E11	Q13765	
P13647	P20700	P27635	P62753	Q14103	O75152	O60884	Q9NW13	Q01780	
Q08211	P09874	P62913	P62424	Q9UKB1	P49368	Q92900	Q8NI27	Q7Z7A3	
Q99569	O60814	P39023	P13637	P35221	P30050	Q15758	O95347	P14678	
P22626	Q92841	Q13263	Q96AG4	P46782	P27348	P14923	P36873	Q2TAY7	
P02538	P50991	P05388	Q13283	Q13200	P05198	P62263	Q9UKM9	Q9BZE4	
P04259	P62805	P11586	P29692	Q13573	Q96C36	Q0VDF9	P08865	P61353	
P08238	Q04695	Q00325	P35998	O15372	Q9Y224	P35269	P04843	Q01105	
P25705	P43243	P51991	Q9Y3I0	Q99729	P62241	P18085	P26038	Q13409	
P08670	Q9UQ80	P51991	Q07065	P62081	Q14152	O43242	Q15424	P46776	
Q86YZ3	Q15046	P13646	P13010	P22695	P32322	P49207	Q9BQG0	P38919	
Q00839	Q8NC51	P04406	P33993	P46777	O43324	Q5JQF8	P05455	Q7Z2W4	
P08779	P52597	P32969	Q8TEB1	O00483	P41091	Q8WU68	Q6Y7W6	P42766	
P09651	Q86V81	P38646	Q01813	P26373	P14866	P42677	Q92947	Q9H1K4	
Q05639	Q9Y265	P12277	P55884	P62701	Q8WVI0	P14618	P15880	P50454	
P10809	O00571	Q9NR30	P62906	Q04637	P35249	P18621	P34897	Q8IX03	
P07900	Q13151	P04181	P38159	P36578	P42704	P30837	Q9BTT0	Q8N9F7	

Table 7.3 Total list of proteins identified in the mass spectrometry of the GST-PS1-Loop FP/VS/AA sample

P49768	Q9Y4U1	Q92947	P39023	Q8TEB1	Q02878	P40616	O43301	P02768
P16152	P11586	P17844	P46777	Q8NE71	P34897	O60884	P61353	Q13363
P09211	P11142	Q12905	Q7Z7L1	P51991	Q14CN4	Q13155	P61221	P41091
O60716	P20700	P04406	Q96EY1	Q15393	O15372	P04637	Q7Z7A3	P26368
P21266	P07814	Q02978	P62805	Q9BUJ2	Q03252	P62241	P36578	P18621
P13645	P61978	P51659	P22695	Q99613	Q96IX5	Q53H96	P62249	Q9Y2X7
P04264	P38646	Q99615	O75643	Q9Y5M8	P62753	Q07021	P62917	Q9BTU6
P28161	Q00610	P46782	P30837	Q12906	P12277	P14625	Q9BY77	P46778
P35527	Q99623	P31689	Q92499	P40429	Q9BRX2	Q9BPW8	P56192	P63244
P68104	P11310	Q15029	P48047	P05198	Q5JPH6	O94813	Q9UI43	Q96PK6
P35908	P07900	Q9NZI8	Q9Y285	Q01081	P0CG48	Q92621	P61619	P09543
P26641	Q9BSD7	P67809	P54136	P62269	P15924	O60282	P50914	P23919
P07437	P17987	Q8NC51	Q9UQ80	P26373	O75616	P42677	P46783	Q9UQE7
P68363	O43175	P26599	Q96C36	Q14103	P29692	P42704	Q01082	P08579
P08107	P24534	P33993	P51570	P11177	P55209	Q9UHR4	P27635	O00233
P68371	Q96S19	Q00325	Q15717	P62424	O94906	Q8N1G4	Q8IX12	Q15424
Q9BVA1	Q9Y265	P12004	P05141	P13010	Q5JTV8	Q09028	Q8IWZ3	P26196
P60709	P50991	P35579	Q96KK5	Q6UWP8	Q16775	P04844	P22830	P50990
Q05639	P06748	P35249	O00571	P78347	O75955	Q9UL03	O95831	P23284
P49411	Q6P2Q9	P04181	P62195	P62906	P81605	P62750	Q92900	P04843
P52272	P43243	P11021	P10619	Q13573	P18085	P83731	P60228	Q8IX03
Q99569	P05388	P38159	Q9NUU7	P11940	Q15120	P48735	O14980	P14678
P25705	Q96I99	P60842	P32969	P27708	Q9UN37	O43324	O15460	Q9NR30
Q08211	P62829	P13674	P35998	Q14152	Q9NTJ3	P43490	Q13765	P22087
P22626	P35232	Q3ZCQ8	P40938	O00148	Q15645	Q04726	Q92979	P13637
P10809	P31943	Q5D862	Q9HAV7	P07686	P35222	O75152	P63208	Q8WWX8
Q9BUF5	P46459	Q9Y3Z3	Q04637	P12956	Q13200	P31327	P68431	P62899
P13647	P49368	P63173	P06576	P35637	Q9H0D6	P17812	P40227	P62280
Q00839	Q9UKB1	O60762	P32322	Q16531	P27816	O00442	Q86TJ2	P15848
O43813	Q92841	P53597	P0C0S5	P23258	Q8IY67	P35269	Q86UK7	Q04656
P02538	P09874	P11172	P23396	Q13435	Q13724	Q8WVI0	Q96GC5	Q02543
P09651	Q9Y2S7	Q9P2J5	P35580	Q9Y5V3	P12236	P35250	Q96E11	Q8ND04
P08670	P19338	Q13263	Q9NZ01	Q01813	Q99729	O43837	Q9BW62	Q9P2R7
P08238	P14868	P52597	P62913	O43143	P62701	Q9H1K4	Q13151	
P41252	Q15046	O43390	Q9P035	P07195	P30050	O75533	Q9BVP2	
P68032	Q9Y230	Q8NI60	Q99714	P55884	P09661	P14866	Q2TAY7	
Q86YZ3	P08779	P18124	P84098	Q8N4T8	O75821	P35268	Q9UBZ9	
P02533	P50454	P07910	Q14204	Q52LJ0	Q8WY22	P26640	O95347	
O15217	P06493	P08559	Q7Z3Y8	O43929	O60814	Q13813	P60866	
O76031	P23246	P47897	Q14203	Q9Y3I0	Q15233	Q9NXW2	Q9BQG0	

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Review

Beyond γ-secretase activity: The multifunctional nature of presenilins in cell signalling pathways

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ABSTRACT

The presenilins are the catalytic subunit of the membrane-embedded tetrameric γ-secretase protease complexes. More that 90 transmembrane proteins have been reported to be γ-secretase substrates, including the widely studied amyloid precursor protein (APP) and the Notch receptor, which are precursors for the generation of amyloid-β peptides and biologically active APP intracellular domain (AICD) and Notch intracellular domain (NICD). The diversity of γ-secretase substrates highlights the importance of presenilin-dependent γ-secretase protease activities as a regulatory mechanism in a range of biological systems. However, there is also a growing body of evidence that supports the existence of γ-secretase-independent functions for the presenilins in the regulation and progression of an array of cell signalling pathways. In this review, we will present an overview of current literature that proposes evolutionarily conserved presenilin functions outside of the γ-secretase complex, with a focus on the suggested role of the presenilins in the regulation of Wnt/β-catenin signalling, protein trafficking and degradation, calcium homeostasis and apoptosis.

Contents

1. Presenilins and the γ-secretase protease complex

The presenilin 1 (PS1) and presenilin 2 (PS2) proteins that form the catalytic core of the γ-secretase protease complex were initially discovered in genetic screens for mutations causing early onset forms of familial Alzheimer's disease (FAD) [1,2]. γ-Secretase is a multisubunit transmembrane proteolytic complex belonging to the family of intramembrane cleaving proteases (ICliPs) of which there are four classes; the serine proteases of the rhomboid class, the metalloproteases in the Site-2-proteases (S2P) class and the GXGD aspartyl proteases, signal peptide peptidases (SPP) and γ-secretase [3]. Of all the ICliPs studied to date γ-secretase is unique as it is a four-protein complex, consisting of the presenilins, anterior pharynx-defective 1 (Aph-1), presenilin enhancer 2 (Pen-2) and Nicastrin, which is only found in multicellular organisms [3,4]. In humans, two forms of presenilin (PS1 and PS2) and two forms of Aph-1 have been identified, one of the Aph-1 homologues is also expressed in two isoforms via alternative splicing, leading to the possible existence of at least six different γ-secretase complexes that may have tissue- or cell type specificity [5]. The presenilins are highly conserved transmembrane proteins that are synthesized as 50 kDa

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holoproteins that undergo endoproteolysis to generate heterodimeric presenilin N-terminal and C-terminal fragments (NTF/CTF), a required activation step in their role as the catalytic subunit of the γ-secretase protease complex [6]. The structure of the PS1-γ-secretase protease complex has revealed the intricate interaction between each subunit and an overall horseshoe-shaped structure composed of the 20 transmembrane domains of presenilin, Pen2, Aph1 and nicastrin [7,8]. To date more than 90 γ-secretase substrates have been identified including APP and a large number of cell surface receptors and adhesion molecules such as Notch, ErbB4, CD44 and E-cadherin [9,10]. The sequential proteolysis of APP culminating in intramembrane cleavage by γ-secretase now serves as a model for the regulated intramembrane proteolysis of numerous transmembrane proteins (Fig. 1) [3,11]. Cleavage of APP by α-secretase or β-secretase results in the release of respective soluble ectodomains (sAPPα or sAPPβ), generation of the APP intracellular domain (AICD) and P3 or Aβ peptides that represent the remaining transmembrane domain of APP [13]. The sAPPα and sAPPβ fragments have been shown to promote neurite growth in cortical neurons in rats and mice and to regulate cell signalling pathways [11,12,14,15], while the AICD fragment has been shown to have a number of cellular functions such as regulating Wnt signalling, gene transcription and apoptosis [16,17].

In general substrates that undergo regulated intramembrane proteolysis are initially cleaved in the extracellular domain by sheddases such as TACE (TNFα converting enzyme) or ADAM (a disintegrin and metalloproteinase domain)/α-secretase, or by aspartyl proteases, such as BACE/β-secretase, before cleavage by the I-CLiP family of proteases [3]. Recently, cleavage of peripheral membrane proteins and proteolysis within substrate ectodomains and luminal loop has been described [18–20]. Furthermore, it has been reported that prerequisite ectodomain shedding may not be required for subsequent cleavage by γ-secretase for some substrates [21]. For several substrates γ-secretase cleavage plays an essential role in a signalling paradigm whereby generation of intracellular domains (ICDs) allows for the spatial segregation of divergent signalling pathways or as is the case for Notch, allows for the translocation of ICDs to the nucleus where they enable transcriptional activation activity [22]. Indeed, *Psen1* knockout animals display a predominant Notch loss-of function phenotype resulting from loss of γ-secretase cleavage of Notch, highlighting the importance of γ-secretase in Notch signalling [23–25]. Cleavage can also be used as a signal for degradation of transmembrane protein fragments and the maintenance of so-called 'membrane proteostasis' [26]. Given the diversity of known γ-secretase substrates the presenilins are proposed to be critically involved in regulating several cell signalling pathways mediated by these molecules, a thorough presentation of which have been reviewed elsewhere [9,10, 22,27–29].

Beyond their role in γ-secretase protease complexes, it has been proposed and in some cases demonstrated that the presenilins have many highly conserved γ-secretase independent regulatory functions in cellular processes and cell signalling, including Wnt signalling, endoplasmic reticulum (ER) calcium homeostasis, as well as lysosomal function and autophagy [29–40]. Evolutionarily many ICliPs are conserved from prokaryotes through to multicellular eukaryotes. Homologues of rhomboids, S2Ps, SPP and γ-secretase have all been identified in animals, plants (*Arabidopsis thaliana and Physcomitrella patens*) and protozoa (*Dictyostelium discoideum*) [27,41–44]. Genetic evidence from presenilin-deficient mice supports this proposal, where mice deficient in *Psen1* and *Psen2* have a more severe phenotype than seen in animals deficient in other γ-secretase components, *Aph-1*, *Pen-2* or *Nicastrin* [45–49]. Similarly, reconstitution studies in *P. patens* revealed that loss of presenilins was associated with distinct phenotypes that could be fully rescued by catalytically inactive presenilin and human presenilin [41], supporting the existence of conserved γ-secretase independent functions for presenilins from plants to humans. This review will focus on current evidence for the existence of γ-secretase independent functions for the presenilins in a diversity of cellular processes; including the regulation of Wnt/β-catenin signalling, calcium homeostasis, cell survival, protein trafficking and degradation.

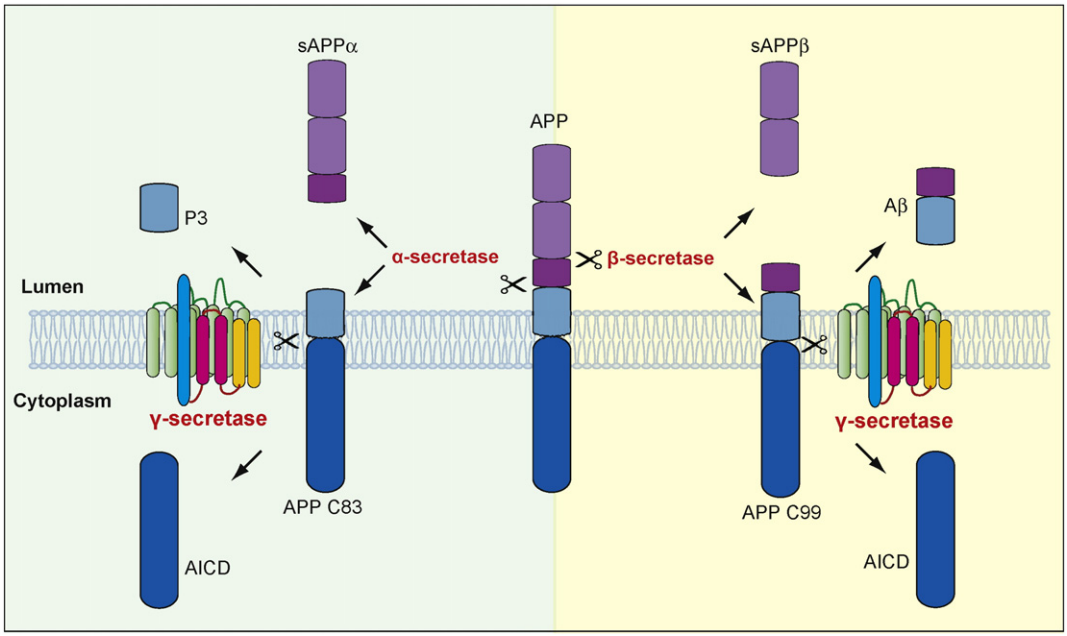

Fig. 1. Regulated intramembrane proteolysis of amyloid precursor protein (APP). In this model the progressive proteolytic cleavage of amyloid precursor protein (APP) is illustrated. Firstly, in the non-amyloidogenic pathway, cleavage in the APP ectodomain by α-secretase produces a soluble ectodomain (sAPPα) and the membrane-anchored APP C83 carboxyl-terminal domain. Next, the γ-secretase complex is recruited to APP-derived C83 and cleaves to generate the P3 fragment and liberate the biologically active APP intracellular domain (AICD). Alternatively, in the amylodiogenic pathway, cleavage of APP by β-secretase produces a soluble ectodomain (sAPPβ) and the membrane-anchored APP C99 carboxyl-terminal domain. Next γ-secretase cleavage of APP C99 liberates the APP intracellular domain (AICD) and generates amyloid-β (Aβ) peptide fragments.

2. Post-translational modification of the presenilins

Compelling evidence for a role for the presenilins in a diversity of γ-secretase-dependent and -independent cell signalling events come from the study of presenilin post-translational modification and from the identification of distinct protein binding domains with the amino acid sequence of the presenilins (Fig. 2). The presenilins undergo endoproteolysis, caspase cleavage, phosphorylation, and ubiquitination, which regulate presenilin function and interaction with numerous proteins [50–52]. Early studies showed that PS1 is phosphorylated by

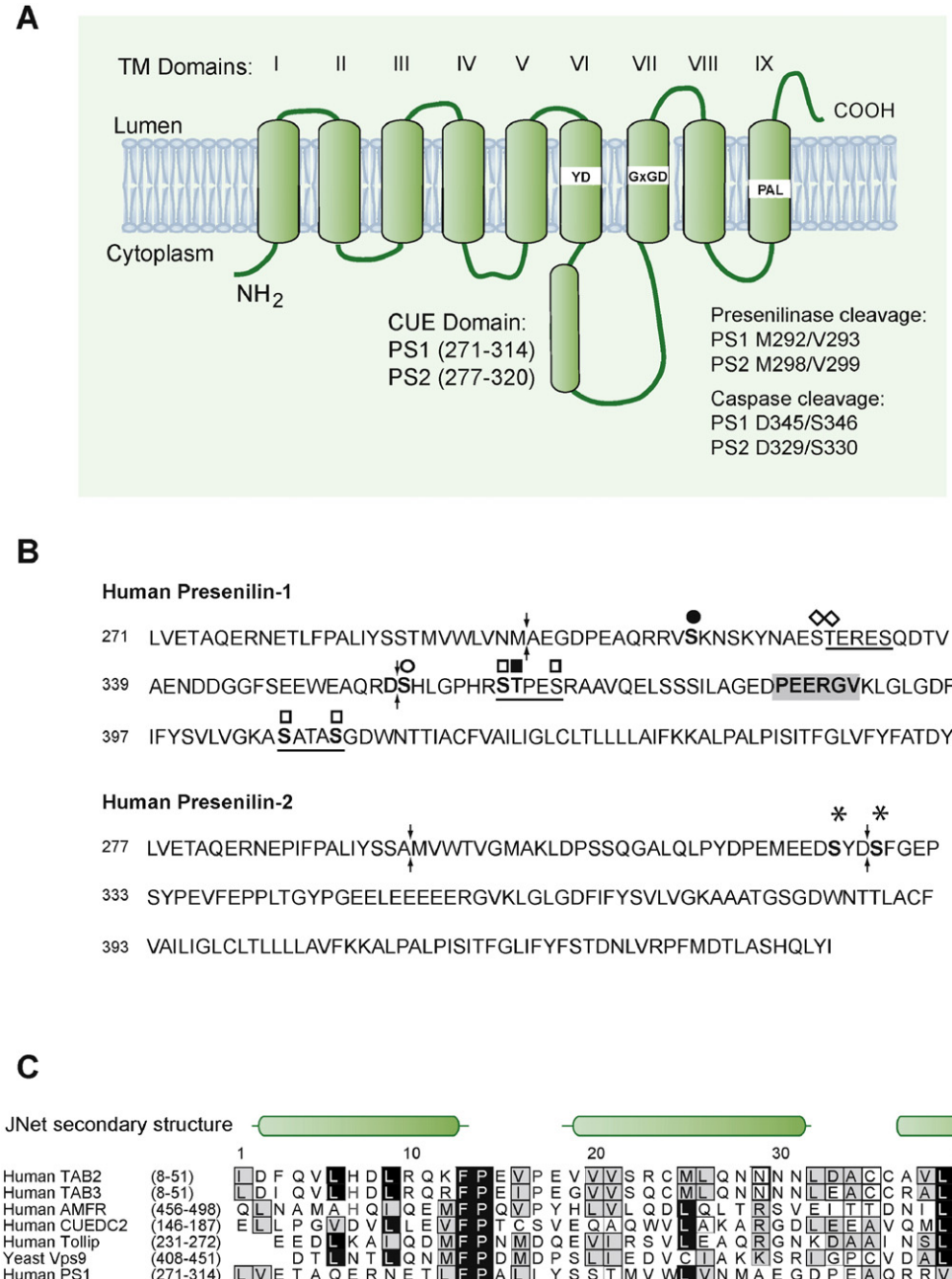

Fig. 2. Presenilin structure, functional domains and post-translational modification. (A), Schematic representation of presenilin-1 (PS1) structure. PS1 contains ten hydrophobic domains arranged in a predominant nine-transmembrane domain [TMD] topology with a large hydrophilic loop domain between TMD six and seven. The protease activity of γ-secretase complexes is mediated by two aspartyl protease active site motifs [YD and GXGD] located in the centre of adjacent TMD 6 and TMD 7 with reverse orientation, and the PAL motifs located in TMD 9. The cytosolic loop contains the presenilinase and caspase cleavage sites, and ubiquitin-binding coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domain mapped as indicated. (B), Previously described covalent post-translational modifications of human presenilins, PS1 and PS2. PS1 contains three GSK3β phosphorylation sites [underlined] whereas PS2 contains none. PS1 and PS2 are subject to serine/threonine phosphorylation by PKA [●], PKC [○], CDK5 [■], GSK3β [□], JNK [◇] AND CK1/2 [⁎] and mapped to specific individual residues. PS1 is also ubiquitinated by Fbw7/Sel-10 and TRAF6 but the site(s) have not been mapped to individual residues. The TRAF6-binding domain (boxed in grey) and the endoproteolysis and caspase cleavage sites are also indicated [arrows]. (C) Sequence alignment of the amino acid sequence of presenilins with several known coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domains [67]. Residues highlighted in black are identical; residues in dark grey are similar; boxed residues are weakly similar. Consensus sequence is shown below the alignment and position of CUE domain FP and LL motifs are indicated with asterisks. The Jnet bioinformatics tool predicted that the putative CUE domains of PS1 and PS2 contain three consecutive α-helices, indicated above sequence alignment. Alignments were carried out with AlignX software.

protein kinase A (PKA) [53], protein kinase C (PKC) [54], glycogen synthase kinase 3β (GSK3β) [55], c-Jun. N-terminal kinases (JNK) [56] and cyclin dependent kinase 5 (CDK5) [53,57] (Fig. 2B), resulting in a variety of biological responses including alterations in γ-secretase activity, [56] Wnt/β-catenin signalling, [58] caspase-3 cleavage of PS1 and PS2 [54], and presenilin stability [57].

It has also been demonstrated that PS1 is ubiquitinated by *Caenorhabditis elegans* SEL-10 [52], Fbw7 the mammalian homologue of SEL-10 [38], and more recently by tumour necrosis factor receptor associated factor 6 (TRAF6), which facilitates Lysine-63 (K63)-linked polyubiquitination of PS1 [50,51]. The ubiquitination of PS1 by *C. elegans* SEL-10 targets PS1 for degradation through the ubiquitin–proteasome system and antagonizes the signalling activity of Notch [52]. Consistent with this, PS1 and Fbw7 interact and regulate the stability and activity of the epidermal growth factor receptor (EGFR) and the Notch ICD (NICD) [38]. The presenilins contain a conserved TRAF6-binding domain [59,60] and undergo TRAF6-mediated K63-linked polyubiquitination, [50,51] which increases PS1 holoprotein stability and function in the regulation of calcium homeostasis, independent of γ-secretase protease activity [50]. Furthermore, the interaction between TRAF6 and PS1 has been shown to promote γ-secretase cleavage of p75 neurotrophin receptor ($p75^{NTR}$) [60], while the loss of TRAF6 E3 ligase activity reduces γ-secretase cleavage of $p75^{NTR}$, TGFβ Type 1 Receptor (TβR1) and interleukin-1 receptor, type 1 (IL-1R1) [51,61]. These data suggests that the interaction between TRAF6, PS1 and γ-secretase substrates plays a role in modulating γ-secretase activity. However, given that TRAF6 can ubiquitinate both PS1 and some γ-secretase substrates, the exact role-played by TRAF6 mediated ubiquitination of PS1 remains to be fully elucidated. What is clear is that these modifications are not only important for the protease assembly and activity of γ-secretase complexes, but are also essential for the stability and activation of presenilins.

The recognition of ubiquitin and polyubiquitin chains by ubiquitin-binding domains (UBDs) is critical for determining the outcome of ubiquitination and subsequent ubiquitin-mediated signalling pathways [62]. To date approximately 20 different UBDs have been identified, including ubiquitin interacting motifs (UIMs), ubiquitin associated domains (UBAs) and the related coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domains [63]. In keeping with a role for ubiquitination in the regulation of presenilin activities, the UBA of ubiquilin is necessary for the binding of ubiquilin to PS1 and PS2 [64], which reduces the formation of NTF/CTF heterodimers by stabilizing presenilin holoproteins within the cell [65]. Ubiquilin transcript variants (TV) 1 and 3 have been shown to cause the accumulation of higher molecular weight forms of PS1 (HMW-PS1) [66]. Ubiquilin is hypothesised to act as a shuttle between ubiquitinated proteins and the proteasome and it has been suggested that the accumulation of HMW-PS1 is a result of weak interactions between Ubiquilin-1 TV3 and the proteasome [66]. PS1 has recently been shown to contain a functional ubiquitin-binding CUE domain (Fig. 2C), which preferentially binds to K63-linked polyubiquitin chains [67]. Interestingly, missense CUE mutants that prevented binding to ubiquitin, do not affect endoproteolysis of PS1, signifying that formation of PS1NTF/CTF heterodimers does not require an intact CUE domain, but suggesting that the presence of the CUE domain may be important for a γ-secretase-independent PS1 holoprotein function. Furthermore, mutation of the CUE domain does not alter PS1-dependent γ-secretase cleave of APP or Notch, nor generation of Aβ40/42 peptides, advocating that the PS1 CUE domain is not necessary for γ-secretase activity [67]. Taken together, these results highlight a function for non-covalent ubiquitin signalling in the regulation of the presenilins and their role in both the γ-secretase-dependent and -independent functions. Further studies will no doubt define the precise regulatory role of post-translational modification in presenilin biology and functions, independent of and dependent upon its role as the catalytic subunit of γ-secretase.

3. Learning from our ancestors

As mentioned earlier, homologues of all γ-secretase components have been identified in plants and protozoans, some of which have emerged as model systems for the study of γ-secretase independent functions of the presenilins [27]. The moss *P. patens* was the first plant species in which conservation of γ-secretase complex components and a γ-secretase independent function for the presenilin homologue (PpPS) and nicastrin was demonstrated [41]. A more recent study in *A. thaliana* similarly showed the conservation of γ-secretase complex components in another plant species [42,43]. Importantly, certain highly conserved amino acid motifs crucial for γ-secretase proteolytic activity, substrate recognition and complex assembly in mammals are similarly conserved in plant species [68]. However, phylogenetic analysis of presenilins indicates that plant and animal homologues fall into two divergent clades [43]. Interestingly, *P. patens* genome contains only one copy of presenilin (PpPS) and does not possess homologues of several mammalian γ-secretase substrates including APP and Notch [41]. In reconstitution studies PpPS was defective in cleavage of a Notch1-based substrate in PS-deficient mouse embryonic fibroblasts (MEFs), but was able to restore normal proliferation rates in PS-deficient MEFs, a function thought to be independent of γ-secretase proteolytic activity [41]. In the phenotypic characterization of a null mutant of presenilin (Ppps), *P. patens* displayed abnormal growth pattern, impaired chloroplast movement and decreased endocytosis [41]. In this regard, in *Arabidopsis* studies employing exogenous expression of γ-secretase subunits impairment of vacuole trafficking was observed [43]. Together this data from two plant species intimates that the predominant function of presenilin in plants is not related to γ-secretase protease activity but perhaps points to an ancestral role for presenilins and the γ-secretase complex in protein endocytosis and trafficking. Consistent with this hypothesis, γ-secretase is present and active in the slime mould *D. discoideum*, [44] where the presenilins have been shown to be essential for *Dictyostelium* cell fate determination and the regulation of phagocytosis. However, in contrast to plants, *Dictyostelium* γ-secretase was still proteolytically active against animal substrates [44]. Again, this suggests an ancestral role for presenilins that extends well beyond what is known about γ-secretase protease activity from predominantly animal studies.

Nevertheless, from animal studies there is also a growing body of evidence showing that the presenilins and γ-secretase may be involved in the regulation of endocytosis, protein trafficking and degradation [69–71], leading to the proposal that signalling functions associated with γ-secretase proteolytic activity and substrate cleavage arose later in evolution [26,44]. In keeping with this, while there are over 90 reported γ-secretase substrates no biological function has been attributed to the majority of these γ-secretase-generated ICDs [10,22]. However, many substrate-derived ICDs are rapidly degraded and support the proposal that the predominant function attributed to γ-secretase would be to facilitate the removal and degradation of membrane bound protein fragments subsequent to ectodomain shedding, thereby acting as a 'membrane proteasome' [72]. Furthermore, mice deficient in Notch signalling or deficient in either of the γ-secretase components, *Aph-1*, *Pen-2* or *Nicastrin*, are able to generate anterior somites, whereas *Psen1* and *Psen2* double-deficient animals are not, suggesting that the presenilins contribute to the generation of anterior somites, independent of their role in γ-secretase [73]. In summary, from an evolutionary perspective, data supports the proposal that presenilins have an important regulatory role in protein endocytosis, trafficking and degradation, which preceded signalling functions associated with γ-secretase substrate cleavage and proteolytic activities.

4. Presenilins in protein trafficking, proteolysis and degradation

An obvious explanation for the role of presenilins and γ-secretase in protein trafficking and degradation is that many reported γ-secretase

substrates are functionally involved in protein transport. For example, members of the mammalian Vsp10p sorting receptor family including sortilin-related receptor with A-type repeat SorLA (also known as SORL1, LR11), Sortilin and SorCS1b are reported to be γ-substrates [74,75]. However, data from several other groups support the proposal that the presenilins can also affect trafficking independent of γ-secretase proteolysis activity. It has been shown that PS1 interacts with Rab11 [76], a small GTPase involved in the regulation of vesicular transport and several other Rab proteins have been reported to be involved in PS1-mediated protein trafficking, such as Rab6 and Rab GDP dissociation inhibitor [77,78]. Consistent with this the presenilins have also been shown to regulate the trafficking and turnover of a number of proteins. For example, presenilin-deficient neurons have been shown to have reduced trafficking of TrkB and EphB receptors to the plasma membrane and diminished neuroprotective qualities of the EphB ligand, efnB [79,80]. Interestingly, this efnB-associated neuroprotection is maintained in cells treated with γ-secretase inhibitors, suggesting a γ-secretase independent function of the presenilins [80]. By contrast PS knockout cells showed an increase in integrin β1 trafficking to the cell surface and an increase in integrin β1 post-translational modification and maturation [81]. These results indicate that the presenilins play a role in chaperoning proteins through the endoplasmic reticulum (ER) to the Golgi independent of presenilins γ-secretase function.

PS1 has also been shown to mediate epidermal growth factor receptor (EGFR) turnover via the endosomal/lysosomal system and as such regulates EGFR signalling [70]. Abnormal EGFR signalling in PS-deficient cells involves γ-secretase independent transcriptional down regulation of the E3 ligase Fbw7 and by affecting a proteasome-dependent ubiquitination step essential for constitutive degradation and stability of EGFR [38]. Additionally, a more recent study has reported that PS1 is necessary for cell-specific transcriptional regulation of EGFR expression and neuroprotection, in a γ-secretase independent manner [82]. The neuronal cell adhesion protein telencephalin also interacts with PS1 and has been shown to increase in half-life and accumulate in autophagic vacuoles in PS-deficient neurons [83]. Telencephalin trafficking was rescued by expression of wild type or catalytically inactive PS1 aspartate mutant and was not affected by γ-secretase inhibitors, demonstrating that this is a γ-secretase independent function of presenilins. Additionally, presenilins have been shown to regulate axonal transport via interactions with GSK-3β thus influencing kinesin-1 and dynein activity and role in transport [84], an activity that was insensitive to γ-secretase inhibitors [85]. Consistent with this, cells expressing PS1 FAD mutations and presenilin conditional knockout (PScDKO) mice show reduced kinesin-1 activity and reduced fast axonal transport (FAT) of type 1 transmembrane receptors [86,87]. Filamin is a protein that is involved in regulating neuronal migration, and overexpression of the PS1 FAD M146L mutant alters filamin distribution within the cell from the cell periphery to the cytoplasm; this alteration is not changed by treatment with a γ-secretase inhibitor [88], suggesting that PS1 regulates filamin localization in a γ-secretase independent manner [89]. Collectively, these observations suggest that the presenilins play an important role in maintaining axonal transport within neurons and in regulating neuronal migration.

PS1 deletion or PS1 FAD mutations have also been shown to disrupt lysosomal acidification and proteolysis, which inhibits autophagy (Fig. 3), a lysosomal degradative pathway for recycling damaged or obsolete organelles and misfolded or aggregated proteins [31,32,83,90–92]. This function is again independent of presenilins' role as the catalytic subunit of γ-secretase [6,31,93]. It has been proposed that PS1 holoprotein serves as a chaperone in the ER for the vATPase V0a1 subunit, a transmembrane component of the proton pump responsible for the acidification of lysosomes [31]. Binding of PS1 in the ER is proposed to stabilize vATPase V0a1 and facilitate its glycosylation, a prerequisite for ER exit [31,94,95]. Oligosaccharyltransferase (OST), a multimeric complex located at the membrane of the ER, transfers a preassembled oligosaccharide to selected asparagine residues within

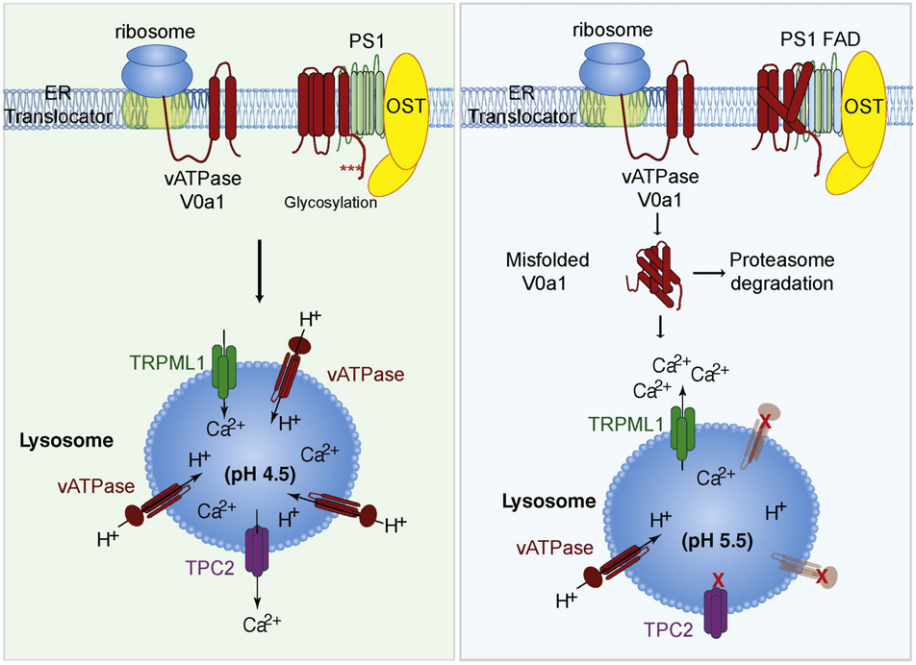

Fig. 3. Presenilins, lysosomal acidification and lysosomal calcium homeostasis. Loss of PS1 or PS1 FAD mutations disturbs lysosomal acidification and proteolysis, which culminate in inhibition of autophagy. Oligosaccharyltransferase (OST), a multimeric complex located at the membrane of the ER, transfers a preassembled oligosaccharide to selected asparagine residues within the consensus sequence asparagine-X-serine/threonine. It is suggested that defects in PS1 leads to compromised glycosylation of vATPase V0a1 subunit by OST, resulting in misfolding and proteasome degradation of the V0a1 subunit, and incomplete lysosomal vATPase assembly and function. Arising from this, disruption to lysosomal acidification (higher lysosomal pH) and degradation occurs leading to cytosolic accumulation of autolysosomes. Defects in PS1 and lysosomal acidification also lead to elevated lysosomal calcium efflux, mediated by pH-regulated transient receptor potential (TRP) cation channel mucoplin subfamily member 1 (TRPML1), and increased cytosolic calcium levels [97]. Increased lysosomal pH also leads to a failure of the endolysosomal NAADP-regulated Ca^{2+} channels, two-pore channel (TPC2), to dissociate from NAADP, rendering the channel inactive and preventing Ca^{2+} efflux from the endolysosomal stores.

the consensus sequence asparagine-X-serine/threonine. In PS1-deficient cells the vATPase V01a subunit is poorly glycosylated and unstable, which prevents proper assembly and function of the multisubunit vATPase pump, resulting in reduced acidification of lysosomes and defective autophagy [31,33]. Consistent with this proposal several lysosome acidification defects and disrupted autophagy have been associates with loss of presenilin or FAD mutations [33,95]. Interestingly, pharmacological inhibition of vATPase induces AD-related autophagy dysfunction and pathologies, while pharmacological normalization of lysosomal acidification in PS1-deficient cells reverses PS1-related defects [96]. In contrast to these studies, others have proposed an alternative model for presenilin function in autophagy, whereby it is proposed that N-glycosylation may not be necessary for the proper trafficking and function of vATPase V0a1 subunit but rather defective glycosylation of the vATPase V0a1 subunit and lysosomal acidification may arise due to disruption in lysosomal calcium storage and release [30]. However, a recent study has demonstrated that disruption in lysosomal calcium storage and release is secondary to dysfunctional lysosomal acidification in PS1-deficient cells [97]. It was demonstrated that abnormal calcium efflux from lysosomes in PS1-deficient cells are caused by a pH-sensitive activation of the endolysosomal calcium channel, transient receptor potential (TRP) cation channel mucoplin subfamily member 1 (TRPML1), which causes elevated cytosolic calcium. Collectively, these data indicate that PS1 deletion or PS1 FAD mutations cause lysosomal and autophagy deficits that contribute to abnormal cellular calcium homeostasis, thereby linking two AD-related pathogenic processes through a single molecular mechanism.

Therefore as it stands, presenilin has a γ-secretase-independent role in regulating protein trafficking, proteolysis and degradation, however given that inhibition of γ-secretase activity can have a negative effect on protein trafficking [69,98], we cannot exclude the possibility that some of the trafficking defects seen in cells with compromised PS1 expression of activity may be attributed to loss or reduced cleavage of Vsp10p sorting receptors or other substrates.

5. Presenilins and calcium homeostasis

Additional to the proposed secondary effects of PS1 on lysosomal calcium storage and homeostasis outlined above, another proposed ancestral role for presenilins relates to the formation of ER calcium leak channels and the regulation of intracellular calcium homeostasis (Fig. 4) [99,100]. Disrupted calcium signalling has been reported to precede the classic AD pathologies, neurofibrillary tangles and Aβ plaques, and is proposed to contribute to neuronal cell death during AD [101]. Similarly, in numerous mouse models of AD increased basal levels of Ca^{2+} have been observed and presenilin FAD mutations have been observed to cause changes in Ca^{2+} levels [93,100,102,103]. There are several lines of evidence supporting a role for presenilins in the regulation of calcium homeostasis of intracellular stores, which appear to be independent of γ-secretase protease activities (Fig. 4). First, the presenilins interact with several proteins functionally involved in calcium signalling, including sorcin, calmodulin, calsenilin, calmyrin and calpain [104–106]. It was next proposed that presenilin holoproteins act as passive Ca^{2+} channels in the ER and those PS FAD mutations alter channel conductance [93]. In an elegant mutagenesis study it was subsequently demonstrated that the hydrophilic catalytic cavity of PS1 facilitates the formation of a calcium leak conductance pore [107]. In parallel, the presenilins have been shown to regulate Ca^{2+} levels through interactions with and activation of Ca^{2+} channels such the sarco/ER Ca^{2+}-ATPase (SERCA) pump [108], the inositol triphosphate receptor ($InsP_3R$) [109], and the Ryanodine receptor (RyR) [110,111]. More recently the presenilins have also been reported to modulate phosphatidylinositol-4,5-bisphosphate (PIP2)-mediated regulation of the transition receptor potential of melastatin related 7 (TRPM7) [112]. Finally, the presenilins have also been proposed to increase the number of contact sites between the ER and mitochondria, thereby facilitating movement of Ca^{2+} from the ER to the mitochondria [113].

Further indicating a γ-secretase-independent role for the presenilins in regulating calcium homeostasis, presenilin-deficient MEFs show a

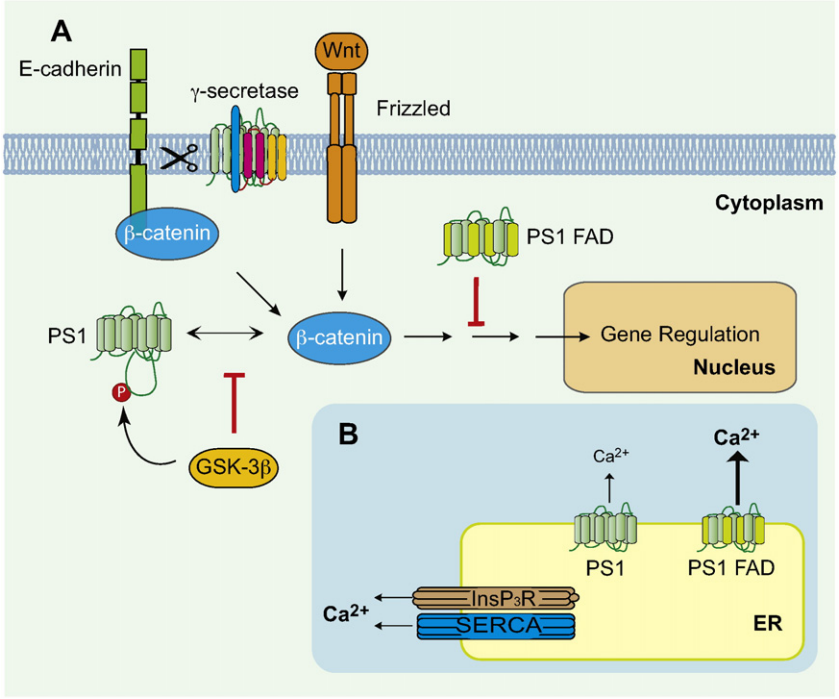

Fig. 4. PS1 and γ-secretase regulate Wnt and calcium signalling within the cell. (A) The γ-secretase cleavage of E-cadherins, facilitates dissociation of β-catenin and α-catenin from the cytoskeleton, thus promoting disassembly of the E-cadherin–catenin adhesion complex. Furthermore, this cleavage releases the E-cadherin ICD to the cytosol and increases the cytosolic pool of β-catenin, a key regulator of the Wnt signalling pathway. PS1 interacts with β-catenin and is a negative modulator of β-catenin/Tcf-4 gene transcription activity, thereby negatively regulating Wnt signalling. This signalling pathway is negatively regulated by the GSK3β phosphorylation of PS1 but is also down regulated by PS1 FAD mutations. (B) The presenilins regulate Ca^{2+} levels within the ER by interacting with the Ca^{2+} channels such as $InsP_3R$ or SERCA. Presenilin FAD mutations have also been shown to increase the level of cytoplasmic Ca^{2+} concentration. The presenilins have also been proposed to act as passive Ca^{2+} channels that release Ca^{2+} from the ER.

decrease in ER Ca^{2+} concentration, which may be as a result of the presenilins regulating $InsP_3R$ levels [114] or SERCA pump levels leading to increased ER [Ca^{2+}] [108], which in both cases normal Ca^{2+} levels can be rescued by overexpression of presenilin holoproteins. Mouse models expressing PS1 FAD mutants show constitutive activation of cAMP response element binding (CREB) gene expression, which is eliminated by inhibition of $InsP_3R$ [115]. Furthermore, PS1 FAD M146V mutant knock-in hippocampal cells showed reduced synaptic plasticity and defects in long-term potentiation (LTP), which can be rescued by inhibition of the RyR [99]. Conditional knockout of presenilins or expression of the PS1 FAD M146V mutant in mouse hippocampal cells has also been shown to increase ER Ca^{2+} levels and enhance susceptibility to induced Ca^{2+} release [100]. These cells also showed an increase in ER RyR levels, which the authors suggest, maybe a result of the decreased presenilin leak channel function, leading to the increased ER Ca^{2+} levels and thus a need for an increase in ER Ca^{2+} channel expression. Additionally, downregulation of PS1 via inhibition of JNK or P53 has been shown to decrease ER Ca^{2+} leakage without altering $InsP_3R$ expression [116]. Consistent with these mammalian studies and supportive of an evolutionary conserved function, X-ray crystallography studies of a presenilin archaeal homologue PSH have shown the presence of a hole that passes through the protein formed from TMD2, TMD3, TMD5 and TMD7, separate to the catalytic core of the enzyme, that is large enough to allow small ions to pass through [117]. Despite all of these studies, some controversies have been raised by reports of the direct measurement of Ca^{2+} ER release from primary neurons, fibroblasts, B-cells and hippocampal neurons [118,119], which showed no difference in ER Ca^{2+} dynamics between cells expressing PS1 WT and PS1 FAD mutants and that any change in ER [Ca^{2+}] in presenilin-deficient cells was not due to increased ER filling [118]. Therefore, while the presenilins have been shown to be important in regulating intracellular Ca^{2+} via several proposed mechanisms, as it stands the Ca^{2+} leak channel function theory of the presenilins remains controversial.

6. Presenilins and Wnt/β-catenin signalling

Several groups have provided substantial data indicating that PS1 acts as a negative modulator of the transcriptional activity of the β-catenin/Tcf-4 complex (Fig. 4) [120–122]. β-Catenin is a multifunctional protein that was first described as a mediator of cadherin-dependent cell adhesion. In adherens junctions β-catenin and the related protein γ-catenin (plakoglobin) are required for recruiting the actin cytoskeleton. In addition to its function in cell adhesion, β-catenin is also central to the Wnt signalling pathway. Therefore, β-catenin is a signalling protein that links transmembrane adhesion proteins, such as the γ-secretase substrate E-cadherin [123], with intracellular signalling pathways and is involved in regulating transcription in a number of anti-apoptotic/survival pathways [124]. When released from the junction complex β-catenin can accumulate in the cytosol and translocate to the nucleus, where it interacts with the Tcf-family of transcription factors and positively regulates expression of several genes involved in development and tumorigenesis. The translocation of β-catenin to the nucleus is controlled by a GSK-3β contained protein complex that regulates the phosphorylation, ubiquitination and degradation of β-catenin [125]. Wnt signalling antagonizes the activity of the degradation complex, which promotes the stabilization and nuclear translocation of β-catenin [126,127]. Early studies identified the presenilins as binding partners for GSK-3β and subsequent works identified three conserved GSK-3β consensus phosphorylation sequences in PS1 and characterized PS1 as an unprimed substrate of GSK-3β [128]. Phosphorylation of PS1 at one of these GSK3β sites (amino acids 353–357) located regulates the interaction between PS1 and β-catenin and phosphorylation at a second site (amino acids 397–401) modulated the stability and degradation of PS1 CTF [129,130]. Phosphorylation by GSK-3β at Ser353 and Ser357 has also been shown to reduce the association of PS1, N-cadherin and β-catenin and a decrease in this association negatively affects Akt signalling [58].

While extensive biochemical data exists supporting a role for presenilins in Wnt/β-catenin signalling, research have proposed numerous mechanisms to support this role, including the interaction of PS1 with E- and N-cadherin and β-catenin and γ-secretase activity [120,131]. It was not until recently that PS1 has been conclusively shown to have both γ-secretase-dependent and -independent roles in β-catenin mediated transcription, where the PS1 holoprotein and the processed PS1 NTF/CTF heterodimers have contrasting roles in modulating β-catenin/Tcf-4 gene transcription [37]. Earlier studies had shown that PS1 FAD mutants inhibit Wnt signalling by increasing β-catenin phosphorylation and degradation [132], and to induce apoptosis in neuronal cells [34]. Additionally, defects in neurite growth observed in FAD murine cells can be reduced by inhibition of β-catenin mediated gene transcription [133]. Others demonstrated that *Drosophila* presenilin (DPS) negatively regulated Wnt signalling [122], and that loss of DPS expression resulted in cytosolic accumulation of armadillo/β-catenin [134]. Consistent with this, conditional knockout of PS1 in keratinocytes resulted in enhanced β-catenin/Tcf signalling and skin tumorigenesis [121]. Some researchers proposed that the enhanced β-catenin/Tcf signalling in presenilin-deficient cells resulted from the delocalization of GSK-3β from the cytoplasm to the endosome [94]. In subsequent studies it has been demonstrated that the inhibitor effect of PS1 holoprotein on β-catenin signalling does not depend on the proteolytic activity of PS1/γ-secretase or on the destabilization of β-catenin [135], but rather a physical interaction between PS1 holoprotein and γ-catenin (plakoglobin) and Tcf-4. Furthermore it was demonstrated that negative effect of PS1 NTF/CTF heterodimers on β-catenin/Tcf-4 transcription was dependent upon γ-secretase activity and proteolysis of E- or N-cadherin and the generation of ICDs, which resulted in destabilization of the β-catenin transcriptional cofactor CBP [37]. In summary, extensive evidence exists to support a γ-secretase independent role for presenilins in Wnt/catenin signalling, however, given that presenilins not only associates with cadherin/catenin complexes, but γ-secretase also cleaves E-cadherin and N-cadherin adds complexity to deciphering the precise regulatory role of presenilins in Wnt signalling [123,131].

7. Presenilins and apoptosis

Apoptosis, also known as programmed cell death, has a critically important role in tissue homeostasis, development, normal ageing and the pathogenesis of several diseases, including cancer and neurological disorders. The presenilins were first linked to apoptosis when cells expressing PS1 FAD mutants were shown to have increased susceptibility to apoptotic stimuli [136–139]. Similarly, expression of PS1 and PS2 in HeLa cells has been shown to cause cell cycle arrest and apoptosis, a process that is increased with the expression of PS1 and PS2 FAD mutants [140–142]. However, primary cortical neurons from PS1 FAD P264L mutant knock-in mice did not show increased neuron degeneration, suggesting that not all FAD mutations promote apoptosis [143,144]. Cells undergo apoptosis through two converging pathways [145]: the extrinsic pathway, mediated by cell surface death receptors, and the intrinsic pathway, which depends upon the release of pro-apoptotic cytochrome-c from the mitochondria (Fig. 5). In the extrinsic pathway, following ligand binding and activation of a death receptor, such as FAS or TNFR1, recruitment of the adaptor Fas-associated protein with death domain (FADD) and pro-caspase-8 leads to the formation of the death-inducing signalling complex (DISC), culminating in the activation of caspase-8, which in turn leads to the activation of effector caspase-3 and caspase-7, resulting in DNA fragmentation and programmed cell death. In the intrinsic pathway, extracellular and intracellular stresses lead to the activation and translocation of the pro-apoptotic Bcl-2 family member Bax to the mitochondria. Bax thereby initiates the release of apoptogenic factors, such as cytochrome-c, resulting in the formation of the cytoplasmic apoptosome, consisting of Apaf-1, pro-caspase-9

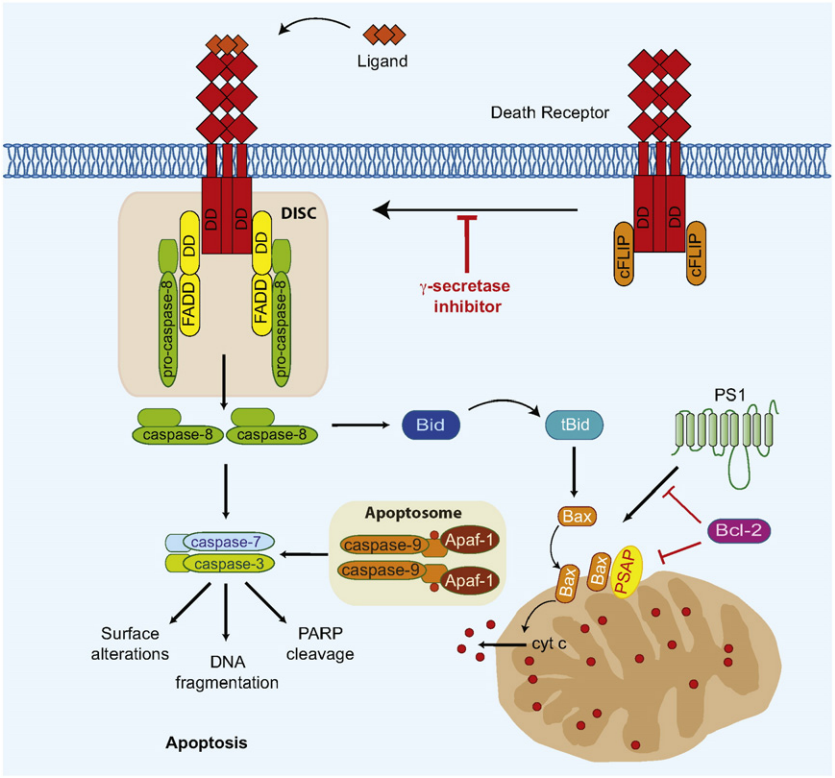

Fig. 5. γ-Secretase-dependent and -independent regulatory role for PS1 in apoptosis. It is proposed that PS1 in a γ-secretase-dependent manner, catalyses the turnover of c-FLIP, promoting DISC assembly and activation of caspase-8, which subsequently activates tBid, resulting in relocalization of Bax, release of cytochrome-c from mitochondria to the cytosol where it binds to Apaf-1, promoting apoptosome formation and caspase-9 activation [153]. In the γ-secretase-independent pathway, it is suggested that PS1 stimulates the formation of a PSAP–Bax complex, resulting in mitochondria dysfunction and release of cytochrome-c to facilitate apoptosome formation and caspase-9 activation. Activated caspase-9 initiates executioner caspases such as caspases-3 and -7 to promote DNA fragmentation and programmed cell death.

and cytochrome-c. Once activated, caspase-9 leads to the activation of caspase-3 and caspase-7. Both pathways can converge through caspase-8-mediated activation of Bid, which subsequently promotes translocation of Bax to the mitochondria. Consistent with a role in cell survival the presenilin proteins are cleaved by caspase-3 during apoptosis to presumably form inactive alternative NTF and CTF fragments for each protein [54,146]. Furthermore, the presenilins have been reported to interact with the anti-apoptotic proteins Bcl-2 and Bcl-X_L and with other apoptosis related proteins such as PS1-associated protein (PSAP), FKBP38, Omi/HtrAS and PARL, though the physiological relevance of these reported interactions remains to be determined [147].

Other studies have demonstrated that increased resistance to apoptosis parallels loss of PS1 expression, while murine B-cells deficient in PS2 show an increased susceptibility to induced apoptosis [148]. Consistent with this, overexpression of PS1 is able to reduce p53-mediated apoptosis in the LTR6 mouse leukaemia cell line [149], while PS2 expression enhances apoptosis via p53 where it increases Bax expression and reduces Bcl-2 expression [150]. This increase in apoptosis is mediated by the PS2 CTF, which increases p53 mediated gene transcription in a γ-secretase independent manner [151]. The presenilin proteins can also affect p53 activity in a γ-secretase dependent manner in that the production of the APP ICD leads to increased p53 expression [152]. Recently efforts were made to define the molecular pathways that mediate the apoptotic effects of PS1, whereby it has been shown that PS1 regulates apoptosis in both γ-secretase-dependent and -independent ways via cellular-FLICE-like inhibitory protein (c-FLIP) and the PS1 associated protein (PSAP), respectively [153]. PSAP has been shown to regulate apoptosis via the intrinsic apoptosis pathway, by controlling the release of cytochrome-c from the mitochondria (Fig. 5) [154]. Silencing of Bax repressed this γ-secretase-independent apoptotic pathway [153], suggesting that the interaction between PS1 and PSAP plays a γ-secretase independent role in regulating the mitochondrial apoptotic pathway. The FKBP38 protein modulates this presenilin-mediated apoptosis, as FKBP38 knockout cells alter Bcl-2 cell localization protecting them from presenilin-mediated apoptosis [155]. Therefore, both presenilin proteins can be seen to play distinct roles in regulating apoptosis, however presenilins may indirectly regulate apoptosis through their association with calcium homeostasis, protein degradation and other pathways, of which the underlying molecular mechanisms are emerging.

8. Concluding remarks

The presenilins are essential parts of the γ-secretase enzyme complex and as such control the regulated intramembrane proteolysis of over 90 transmembrane proteins. These cleavage events play important roles in cellular differentiation, gene transcription and disease progression. In addition to its γ-secretase-dependent functions the presenilins have a number of γ-secretase independent roles in Wnt signalling, calcium homeostasis, protein trafficking and degradation. These γ-secretase independent roles allow the presenilins another means to regulate cell signalling and gene transcription through pathways such as the β-catenin or Akt pathways and also to affect apoptosis and the inflammatory response. Our understanding of the apparent broad function of presenilins and γ-secretase in biological processes has grown significantly in the past decade and further studies will no doubt define the precise role of presenilins, independent of and dependent upon its role as the catalytic subunit of γ-secretase.

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A ubiquitin-binding CUE domain in presenilin-1 enables interaction with K63-linked polyubiquitin chains

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ABSTRACT

The presenilins (PS1 and PS2) are the catalytic component of the γ-secretase intramembrane protease complex, involved in the regulated intramembrane proteolysis of numerous type I transmembrane proteins, including amyloid precursor protein (APP) and Notch. Herein, we describe the identification and characterization of a CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation) ubiquitin-binding domain (UBD) in PS1, and demonstrate that the CUE domain of PS1 mediates non-covalent binding to Lysine 63-linked polyubiquitin chains. Our results highlight a γ-secretase-independent function for non-covalent ubiquitin signaling in the regulation of PS1, and add new insights into the structure and function of the presenilin proteins.

1. Introduction

The reversible covalent attachment of the 8kDa protein ubiquitin (Ub) to cellular proteins, predominantly to primary amines (ε-amino groups of Lys and to the N termini of proteins) targets proteins for proteasomal degradation and also facilitates several non-proteasomal functions in the assembly, amplification and transmission of intracellular signals [1–4]. Protein ubiquitination is the culmination of a multistep process involving three classes of enzymes, known as ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2s), and substrate-specific ubiquitin protein ligases (E3) [4]. Proteins may be ubiquitinated on a single lysine, resulting in monoubiquitination or on several lysine residues resulting in multiubiquitination. In addition, some E2/E3 combinations can then use lysines on the substrate-conjugated ubiquitin to act as acceptors during sequential rounds of ubiquitination, resulting in substrate polyubiquitination [5]. Ub contains seven lysines, which can be utilized during polyubiquitin chain formation and depending on which of the seven lysines act as the acceptor, the ubiquitin chains will have different types of linkages with diverse conformations and create a diversity of molecularly distinct signals in the cell [4,6]. The range of substrate-ubiquitin structures is important for the targeting of ubiquitinated substrates to different fates. For example, K11- and K48-linked polyubiquitin chains generally target proteins for proteasomal degradation [7], while K63-linked chains can regulate kinase activation, DNA damage tolerance, signal transduction, and endocytosis [8,9].

Modulation of protein–protein interactions is an important mechanism involved in the assembly, amplification and transmission of intracellular signals. The recognition of ubiquitin and polyubiquitin chains by ubiquitin-binding domains (UBDs) is critical for determining the outcome of ubiquitination and subsequent ubiquitin-mediated signaling pathways [2]. To date, approximately 20 different UBDs have been identified, including ubiquitin interacting motifs (UIMs), ubiquitin associated domains (UBAs) and the related coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domains [10–12]. The UBA and CUE domains are similar in size, approximately 40 residues, and show a common structural homology consisting of a three helical bundle [10]. The CUE domain was initially characterized as an ubiquitin-binding motif and named for the yeast Cue1p protein, which is essential for the targeting of ubiquitinated protein to degradation pathways [13–15]. Functional studies subsequently revealed that CUE domains promote the ubiquitination of the proteins that

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contain it [15]. Since then, CUE domains have also been reported to contribute to the stability and specificity of the CUE–ubiquitin complex, and moreover that CUE domain containing proteins play an important role in stabilizing its binding partner [16,17]. The CUE domain of other proteins has been shown to mediate interactions between ubiquitin and CUE domain containing proteins, which facilitates their monoubiquitination [18–20].

The presenilins, PS1 and PS2, are highly conserved transmembrane proteins that are synthesised as 50kDa holoproteins that undergo endoproteolysis to generate heterodimeric presenilin N-terminal and C-terminal fragments (NTF/CTF) that form the catalytic subunit of the γ-secretase protease complex. Initially characterized as a protease responsible for the cleavage of amyloid precursor protein (APP) and the generation of amyloid-β peptides, the γ-secretase protease is now known to be responsible for the cleavage of numerous type I transmembrane proteins associated with several developmental and cellular processes, including the Notch receptor, ErbB4, Cadherins, Insulin-like growth factor receptor (IGFR1) [21] and the interleukin-1 receptors, IL-1RI [22,23] and IL-1RII [24]. In addition to the well-researched role of presenilins as the catalytic core of the γ-secretase protease, the involvement of presenilin holoproteins in the regulation of intracellular calcium homeostasis has become a focus of presenilin and Alzheimer's disease research [25–28].

Critical to the complexity of presenilin-associated activities, PS1 and PS2 are differentially controlled by post-translational modifications including endoproteolysis, caspase cleavage, phosphorylation and ubiquitination [29]. These modifications determine subcellular localisation [30], selectivity of binding-partners, rate of protein turnover [31] and γ-secretase protease activity [32,33]. PS1 is ubiquitinated by SEL-10 and tumor necrosis factor receptor associated factor 6 (TRAF6), which regulate γ-secretase activity and calcium homeostasis [34], respectively. Here we report the identification and characterization of a ubiquitin binding CUE domain in PS1, which facilitates the binding of PS1 to K63-linked polyubiquitin chains.

2. Materials and methods

2.1. Cell culture and transfection

HEK293T and presenilin-deficient murine embryonic fibroblasts (MEFs) cells were maintained in Dulbecco's modified Eagle's medium (DMEM-21) supplemented 10% fetal bovine serum at 37 °C. Transfection of HEK293T and MEFs was performed using the calcium phosphate precipitation method and TurboFect™ (BioRad Laboratories), respectively.

2.2. Expression vectors construction

The pcDNA3.1-PS1 expression construct was described previously [35,36]. PS1ΔCUE, PS1F283A/P284A and PS1V309A/S310A, were generated by site-directed mutagenesis using QuickChange Site Directed Mutagenesis Kit (Stratagene) and the following primers: PS1ΔCUE: GATTTAGTGGCTTATAATGCAGAA AGCACAGAA (sense) and TTCTGCATTATAAGCCACTAAATCATATAC TGA (antisense); PS1: CGGGGTACCGCCATGACAGAGTTACCTGCA CCGTTGTCC (sense) and CCGGAATTCCTAGATATAAAATTGATGGA ATGCTAATTG (antisense); PS1F283A/P284A: GAGAAATGAAACGCT TGCTGCAGCTCTCATTTACTCC (sense) and GGAGTAAATGAGAG CTGCAGCAAGCGTTTCATTTCTC (antisense); PS1V309A/S310A: GGAAGCTCAAAGGAGAGCAGCCAAAAATTCCAAG (sense) and CTTGGAATTTTTGGCTGCTCTCCTTTGAGCTTCC (antisense). Position of mutated sites are underlined. GST-fusion proteins, GST-PS1 loop domain (residues 265–380), GST-PS1F283A/P284A and GST-PS1V309A/S310A loop domain mutants were amplified by PCR (KOD polymerase, Novagen) using CGGGGATCCGCCGTTTT GTGTCCGAAAGGT (sense) and CCGGAATTCCTATTTTACTCCCCTTT CCTC (antisense) and subcloned into pGEX-6P-1 GST expression vector (GE Healthcare). HA-ubiquitin, HA-ubiquitin $K48_{only}$ and HA-ubiquitin $K63_{only}$ were a generous gift from Dr. R. Carmody (University of Glasgow). HA-P62 was a gift from Prof. Jorge Moscat & Dr. Marie Wooten (Sanford Burnham Medical research Institute, CA), and the HA-P62 F406V mutant was generated by site-directed mutagenesis using previously published primer pairs [37]. The NEXT (Notch extracellular truncation) expression plasmid was a gift from Raphael Kopan (Washington University) [38]. APP-CT100 was a gift from Scios Inc.

2.3. Western blot analysis

Total or immunoprecipitated protein extracts were obtained from cells 24–48 h after transfection with specific plasmids. Cells were lysed (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM sodium orthovanadate and protease inhibitor mixture (Complete™, Roche Molecular Biochemicals)) and protein concentrations in the extracts were measured with a bicinchoninic acid assay (Pierce). Equal amounts of extracts were resolved on 6%, 10%, 12% or 15% SDS–PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with anti-PS1 NTF [39], anti-PS1-NTF (Chemicon), anti-HA (Covance), anti-APP (Sigma Aldrich), anti-Ubiquitin (P4D1, Santa Cruz Biotechnology), anti-β-actin (Sigma Aldrich) and anti-cleaved Notch 1 (Val 1744) (Cell Signaling Technology) antibodies, followed by incubation with secondary horseradish peroxidase-labeled anti-mouse, anti-rabbit (Dako), or infrared secondary antibodies IRDye® 800 Goat Anti-Rabbit IgG or IRDye® 800CW Goat Anti-Mouse IgG (Licor Biosciences).

2.4. In vitro ubiquitin binding assay

For the whole-cell ubiquitin binding studies, cells expressing the indicated proteins were lysed under stringent denaturing conditions and immunopurified with anti-P62, or PS1 antibodies and protein G-sepharose beads (Invitrogen) and subsequently washed 3 times with covalent buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton, 0.1% SDS, 0.5% sodium deoxycholate, 15 mM NEM and protease inhibitors) and once with binding buffer (20 mM Tris–HCl (pH 7.6), 50 mM NaCl, 0.1% NP40, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Samples were incubated overnight at 4 °C in binding buffer with 5 μg of polyubiquitin (K48- or K63-linked) (Boston Biochem), washed with binding buffer and loaded onto a 10% SDS–PAGE, recombinant polyubiquitin (K48- or K63-linked) (1 μg) was used as a positive control, followed by Western blotting analysis using an antibody directed against ubiquitin (P4D1, Santa Cruz Biotechnology).

For the in vitro ubiquitin binding assays, recombinant (20 μg) GST-PS1 loop domain, GST-PS1F283A/P284A or GST-PS1V309A/S310A loop domain mutants were immobilized onto glutathione agarose beads (Sigma Aldrich) and incubated with 800 μg total protein extracted from HEK293T cells, either non-transfected or overexpressing HA-Ub-$K48_{only}$ or HA-Ub-$K63_{only}$ lysed in binding buffer. Samples were incubated overnight at 4 °C, washed in binding buffer and loaded onto SDS–PAGE followed by Western blotting analysis with an antibody directed against ubiquitin (P4D1, Santa Cruz Biotechnology).

2.5. Expression and purification of GST-tagged recombinant proteins

The pGEX-6P-1 GST-PS1 loop domain (residues 265-380), pGEX-6P-1 GST-PS1F283A/P284A and pGEX-6P-1 GST-PS1V309A/

S310A loop domain mutant expression plasmids were introduced into the *Escherichia coli* strain BL21-CodonPlus (DE3)-RIL (Stratagene) for recombinant protein expression. Induction of expression, glutathione agarose affinity chromatography and purification of the recombinant proteins was performed using previously established methods [40]. The purity and molecular weight of the recombinant protein samples were analyzed by SDS–PAGE. The protein concentration was determined from UV absorbance at 280 nm.

2.6. ELISA for Aβ40 and Aβ42

HEK293T cells were transfected by calcium phosphate precipitation with indicated constructs. Thirty-six hours after transfection, cell culture medium was collected for ELISA analysis and cells were subjected to Western blotting. ELISA kits for Aβ40 and Aβ42 were purchased from Invitrogen (#KHB3481 and #KHB3441). ELISA analyses were carried out according to the manufacturer's protocol.

2.7. Statistical analysis

Microsoft Excel was used for the statistical analysis. Data are shown as means + standard error (S.E.M.) of replicate samples in at least three independent experiments. Student's *t*-test was used to test comparisons between two groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Presenilins contain a highly conserved putative CUE ubiquitin-binding domain

To further examine the posttranslational modification of presenilins, the amino acid sequences of PS1 and PS2 hydrophilic loop domains were analyzed by sequence analysis. This revealed a low-homology between the hydrophilic loop domain of PS1 and the CUE ubiquitin-binding domain of several proteins (Fig. 1). A sequence alignment of the hydrophilic loop domain of PS1 and PS2 with several known CUE domains revealed a high conservation of a methionine–phenylalanine–proline and to a lesser extent a di-leucine motif, characteristic of CUE ubiquitin-binding domains, as well as a number of other hydrophobic residues thought to be important for non-covalent interaction with ubiquitin (Fig. 1B). A sequence alignment of this region of PS1 among several species demonstrates strong evolutionary conservation of the sequence surrounding and including the phenylalanine–proline motif and a weaker conservation of the sequence containing the PS1 valine–serine sequence which corresponds to the position of the di-leucine motif, present in some, but not all CUE domains (Fig. 1C). The CUE

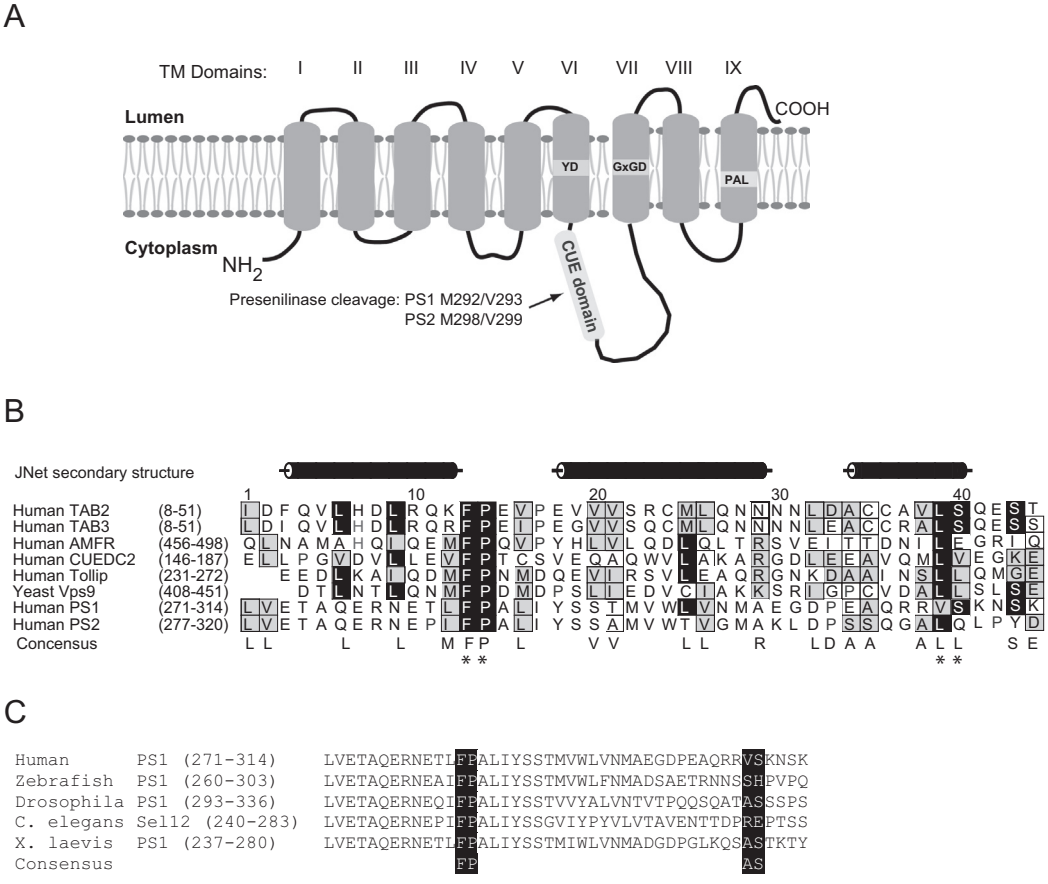

Fig. 1. Presenilins contain a putative CUE ubiquitin-binding domain. (A) Schematic of the Presenilin-1 (PS1) protein indicating the proposed transmembrane domains, hydrophilic loop domain and putative CUE domain. (B) A sequence alignment of the amino acid sequence of presenilins with several known CUE domains. Residues highlighted in black are identical; residues in dark gray are similar; boxed residues are weakly similar. Consensus sequence is shown below the alignment and position of CUE domain FP and LL motifs are indicated with asterisks. The JNet bioinformatics tool predicted that the putative CUE domains of PS1 and PS2 contain three consecutive α-helices, indicated above sequence alignment. (C) A sequence alignment of the hydrophilic loop domain of PS1 from several species demonstrates high conservation of this region. Highly conserved amino acid residues known to be important for non-covalent interaction with ubiquitin are highlighted. Alignments were carried out with AlignX software.

domain is structurally related to the ubiquitin binding UBA domain and is comprised of a three-helix bundle. Using JNET, www.compbio.dundee.ac.uk [41], secondary protein structure prediction software, the putative CUE domains of PS1 and PS2 were predicted to contain three consecutive α-helices, with the conserved methionine–phenylalanine–proline residues located close to α-helix 1 and the PS1 valine–serine sequence residing in αhelix 3 (Fig. 1B). Given that this sequence conservation and predicted structural arrangement are shared by the putative CUE domain of presenilins and several other CUE domains, it supports our hypothesis that the newly identified CUE domain in presenilins may mediate interactions between presenilins and ubiquitinated substrates.

3.2. Presenilins non-covalently interact with K63-linked polyubiquitin

To determine whether presenilins interact non-covalently with ubiquitin and contain functional CUE ubiquitin-binding domains, HEK293T cells were transfected with pcDNA3.1, pcDNA3.1-PS1 or pcDNA3.1-PS1ΔCUE (Fig. 2A), and 24 h post-transfection cells were harvested under denaturing conditions and the cell lysates were immunoprecipitated with an anti-PS1 antibody. The immunoprecipitates were then incubated overnight with recombinant lysine 63 (K63)-linked or lysine 48 (K48)-linked polyubiquitin and after stringent washing ubiquitin-binding was determined by western blotting. Our data demonstrates that PS1 has a preference

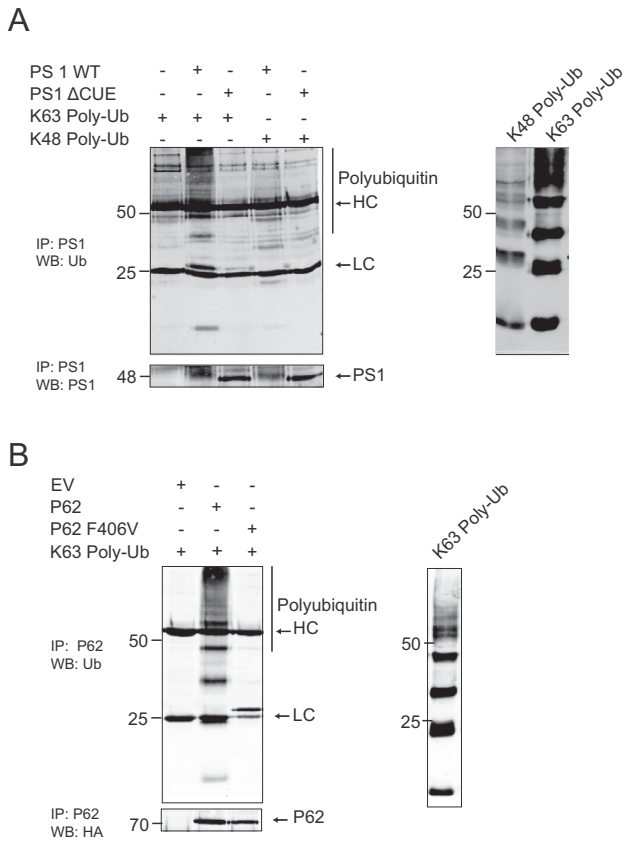

Fig. 2. Presenilins selectively interact with K63-linked polyubiquitin. HEK293T cells expressing wild type or mutant (A) PS1 (B) P62 were lysed under stringent denaturing condition, immunopurified and incubated with 5 μg of recombinant K48-linked or K63-linked polyubiquitin, and bound proteins resolved by SDS–PAGE and immunoblotted with antibodies against ubiquitin (PD41), PS1 or P62. Loading control for recombinant lysine 63 (K63)-linked (1 μg) or lysine 48 (K48)-linked polyubiquitin (1 μg) used in experiments is also indicated (right panels). IP, immunoprecipitated; WB, Western blot; HC, IgG heavy chain; LC, IgG light chain.

for binding K63-linked over K48-linked polyubiquitin chains (Fig. 2A). In contrast, no binding to K63-linked polyubiquitin to PS1 was observed in the PS1ΔCUE mutant. Loading control for recombinant lysine 63 (K63)-linked or lysine 48 (K48)-linked polyubiquitin used in experiment is indicated (Fig. 2A, right panel). To validate the in vitro ubiquitin-binding assay employed in these studies, the ability of PS1 to bind polyubiquitin was compared to that of P62/sequestosome 1, which contains a functional UBA domain that binds to K63-linked polyubiquitin [42]. A single point mutation within the UBA domain of P62, P62F406V, abolishes ubiquitin binding to the P62 UBA domain. Consistent with previous studies, wild type P62 but not P62F406V bound to K63-linked polyubiquitin (Fig. 2B). Loading control for recombinant lysine 63 (K63)-linked polyubiquitin used in experiment is indicated (Fig. 2B, right panel). Together these results demonstrate that PS1 contains a functional ubiquitin-binding domain that has a preference for binding K63-linked polyubiquitin.

Based on sequences predicted to be important for ubiquitin binding to other CUE domains, we used a site-directed mutagenesis approach to generate mammalian expression constructs of the PS1 CUE missense mutants PS1F283A/P284A and PS1V309A/S310A. Previous studies demonstrated that mutation of the conserved methionine–phenylalanine–proline and di-leucine motifs reduce the ability of the CUE domain of specific proteins to interact with ubiquitin [43]. JNet secondary structure prediction analysis revealed that the triple helical super-secondary structure of the CUE domain should not be disrupted by these mutations (data not shown). HEK293T cells were transiently transfected with equal quantities of pcDNA3.1 (Vector), wild type PS1, PS1ΔCUE, PS1F283A/P284A or PS1V309A/S310A. The PS1 proteins were isolated using immunoprecipitation and were then incubated with recombinant K63-linked polyubiquitin (Fig. 3A). Western blotting analysis revealed that wild-type PS1 and the PS1 F283A/P284A mutant bound to ubiquitin, while in contrast the PS1ΔCUE and PS1V309A/S310A mutants had reduced interaction with K63-linked polyubiquitin (Fig. 3A). These results suggest that the K63-linked polyubiquitin binding capability of the putative CUE of PS1 is mediated by the VS sequence, while the FP motif does not appear to be as important in PS1 binding to K63-linked polyubiquitin as it shows a similar level of ubiquitin-binding as wild-type PS1.

To further examine the ubiquitin binding ability of the PS1 CUE domain, three GST-tagged recombinant proteins were created: a GST-PS1 loop domain protein (residues 265–280) containing the CUE domain, GST-PS1F283A/P284A and GST-PS1V309A/S310A loop domain mutants. The recombinant proteins were bound to glutathione agarose beads and then incubated with cell lysate from cells overexpressing HA-ubiquitin $K63_{only}$, a ubiquitin mutant that can only form K63-linked polyubiquitin chains within cells [44]. GST protein was used as negative control for this experiment. The resulting samples were run on a 10% SDS–PAGE gel and the presence of bound ubiquitin was detected by immunoblotting for HA. Consistent with data presented (Fig. 3A), the GST-PS1 loop domain fusion protein and GST-PS1F283A/P284A mutant bound to K63-linked polyubiquitin, while the GST-PS1V309A/S310A loop domain mutant lost the ability to bind K63-linked polyubiquitin (Fig. 3B). To verify PS1 selectivity for binding to K63-linked ubiquitin in our in vitro assay, next, recombinant GST-PS1 loop domain protein and GST-PS1V309A/S310A mutant were bound to glutathione agarose beads and then incubated with cell lysate from cells overexpressing ubiquitin $K48_{only}$ or ubiquitin $K63_{only}$, (Fig. 3C). Again, the GST-PS1 loop domain fusion protein selectively bound to K63-linked polyubiquitin and not K48-linked polyubiquitin, while the GST-PS1V309A/S310A mutant lost the ability to bind K63-linked polyubiquitin (Fig. 3C).

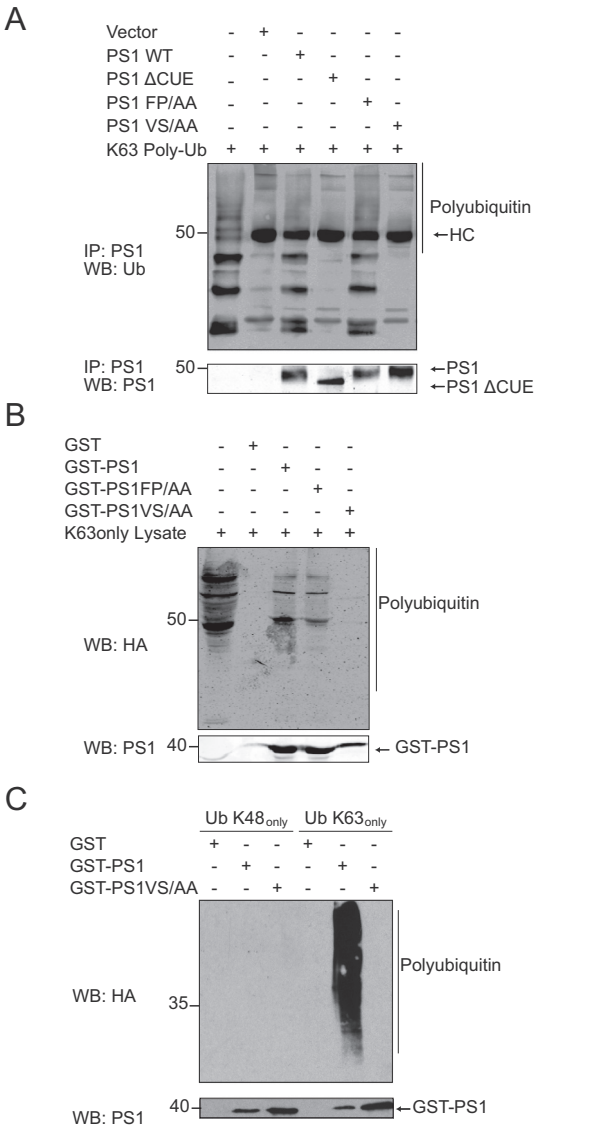

Fig. 3. Disruption of the PS1 CUE domain impairs binding to K63-linked polyubiquitin. (A) HEK293T cells expressing pcDNA3.1 (Vector), wild type PS1, PS1ΔCUE, PS1F283A/P284A or PS1V309A/S310A were lysed under stringent denaturing conditions, immunopurified with an anti-PS1 antibody and incubated with 5 μg of recombinant K63-linked polyubiquitin, and bound proteins resolved by SDS–PAGE and immunoblotted with antibodies against ubiquitin (PD41) or PS1. (B) Bacterially purified recombinant GST, GST-PS1 (loop domain), GST-PS1F283A/P284A and GST-PS1V309A/S310A were conjugated to glutathione agarose beads and incubated with cell lysates from HEK293 cells transiently expressing HA-Ub-$K63_{only}$ expression plasmid. Bound proteins were resolved by SDS–PAGE and immunoblotted with antibodies against HA or PS1. (C) Bacterially purified recombinant GST, GST-PS1 (loop domain) and GST-PS1V309A/S310A mutant were conjugated to glutathione–agarose beads and incubated with HEK293 cell lysates from cells transiently expressing either HA-Ub-$K48_{only}$ or HA-Ub-$K63_{only}$ expression plasmid. Bound proteins were resolved by SDS–PAGE and immunoblotted with antibodies against HA or PS1. IP, Immunoprecipitated; WB, Western blot; HC, IgG heavy chain.

3.3. Presenilins CUE domain is dispensable for γ-secretase activity

Next we determined if the CUE domain was required for presenilin endoproteolysis and γ-secretase activity. Given that the CUE domain (residues 271–314) of PS1 is located within the large hydrophilic loop domain and spans the sites of presenilin endoproteolysis (PS1 Met292/Val293) [32,45], we examined the possible involvement of the putative CUE domain in endoproteolysis of PS1 by deletion of the entire CUE domain from PS1 (PS1ΔCUE) and mutagenesis of the two conserved motifs within the PS1 CUE domain (PS1F283A/P284A and PS1V309A/S310A). Presenilin double knockout murine embryonic fibroblasts were transfected with expression constructs directing the synthesis of wild-type PS1, catalytically inactive PS1D257A/D385A (PS1ΔAsp) mutant, CUE domain deletion mutant PS1ΔCUE, or CUE domain point mutants PS1F283A/P284A or PS1V309A/S310A. Cell lysates were immunoblotted with anti-PS1 NTF antibody (Fig. 4A). As anticipated, expression of the catalytically inactive PS1D257A/D385A (PS1ΔAsp) mutant, or deletion of PS1 CUE domain prevented the endoproteolysis of PS1, and abolished the formation of PS1 endoproteolytic NTF fragment (Fig. 4A). However, expression of the PS1 CUE domain point mutants PS1F283A/P284A and PS1V309A/S310A had no effect on PS1 endoproteolysis and formation of PS1 NTF fragment.

Presenilin holoprotein undergoes endoproteolysis into NTF/CTF heterodimers and has been predominantly studied as a component of the γ-secretase protease complex involved in the regulated intramembrane proteolysis of APP, Notch and several other type I integral membrane proteins [46]. We next measured γ-secretase activity in cells expressing CUE mutants by studying the cleavage of a truncated APP mutation (APP CT100-FLAG), which corresponds to the β-secretase generated APP C99 C-terminus fragment, which is a constitutive substrate for γ-secretase protease. First, HEK293T cells, were transiently transfected with APP CT100-FLAG plasmid and co-transfected with pcDNA3.1 (Vector), PS1, PS1ΔCUE PS1F283A/P284A or PS1V309A/S310A and cell lysates were subjected to Western blot analysis with an anti-APP C-terminus-specific antibody (Fig. 4B). In cells expressing PS1, overexpressed APP C99-FLAG fragment is clearly detected (Fig. 4B, lane 3). In cells expressing PS1 and treated with the γ-secretase inhibitor, Compound E, accumulation of overexpressed APP C99-FLAG and endogenous APP C99 fragments are clearly detected (Fig. 4B, lane 4). In contrast, expression of PS1 CUE mutants did not alter levels of exogenous APP C99-FLAG nor endogenous APP C99 C-terminal fragment, which indicates that CUE domain deletion does not induce a major change in γ-secretase activity (Fig. 4B). To further confirm the result with a more sensitive method, cell culture medium was collected after transfection and tested using ELISA to measure the concentration of Aβ40 and Aβ42 (Fig. 4C). Results showed that CUE domain deletion or mutagenesis does not induce significant change in the generation of soluble Aβ40 and Aβ42. To further validate this observation, PS1-deficient MEFs were transiently transfected with pcDNA3 (Vector), PS1, PS1ΔCUE PS1F283A/P284A or PS1V309A/S310A and cell lysates were analyzed for endogenous APP C99, by Western blotting with an APP C-terminus-specific antibody (Fig. 4D). Again, expression of the CUE mutants had no effect on levels of endogenous APP C99, whereas treatment with the γ-secretase inhibitor Compound E (lane 3) caused the accumulation of the APP C99 fragment, again demonstrating that loss of or mutagenesis of the PS1 CUE domain does not significantly alter γ-secretase cleavage of APP.

To further examine the functionality of the PS1 CUE domain in the cleavage of γ-secretase substates. HEK293T cells, were transiently transfected with NEXT, a Notch construct that is constitutively cleaved by γ-secretase, and co-transfected with pcDNA3 (Vector), wild-type PS1, PS1ΔCUE, PS1F283A/P284A or PS1V309A/S310A. Twenty-four hours post-transfection cell lysates were prepared and subjected to Western blot analysis with an anti-cleaved Notch 1 specific antibody (Fig. 4E). In cells expressing PS1, robust generation of NICD was evident (Fig. 4E, lane 3), while in cells treated with the γ-secretase inhibitor, compound E (CpdE) (Fig. 4E, lane 4) formation of NICD was inhibited. In contrast, in cells expressing PS1ΔCUE, PS1F283A/P284A or PS1V309A/S310A,

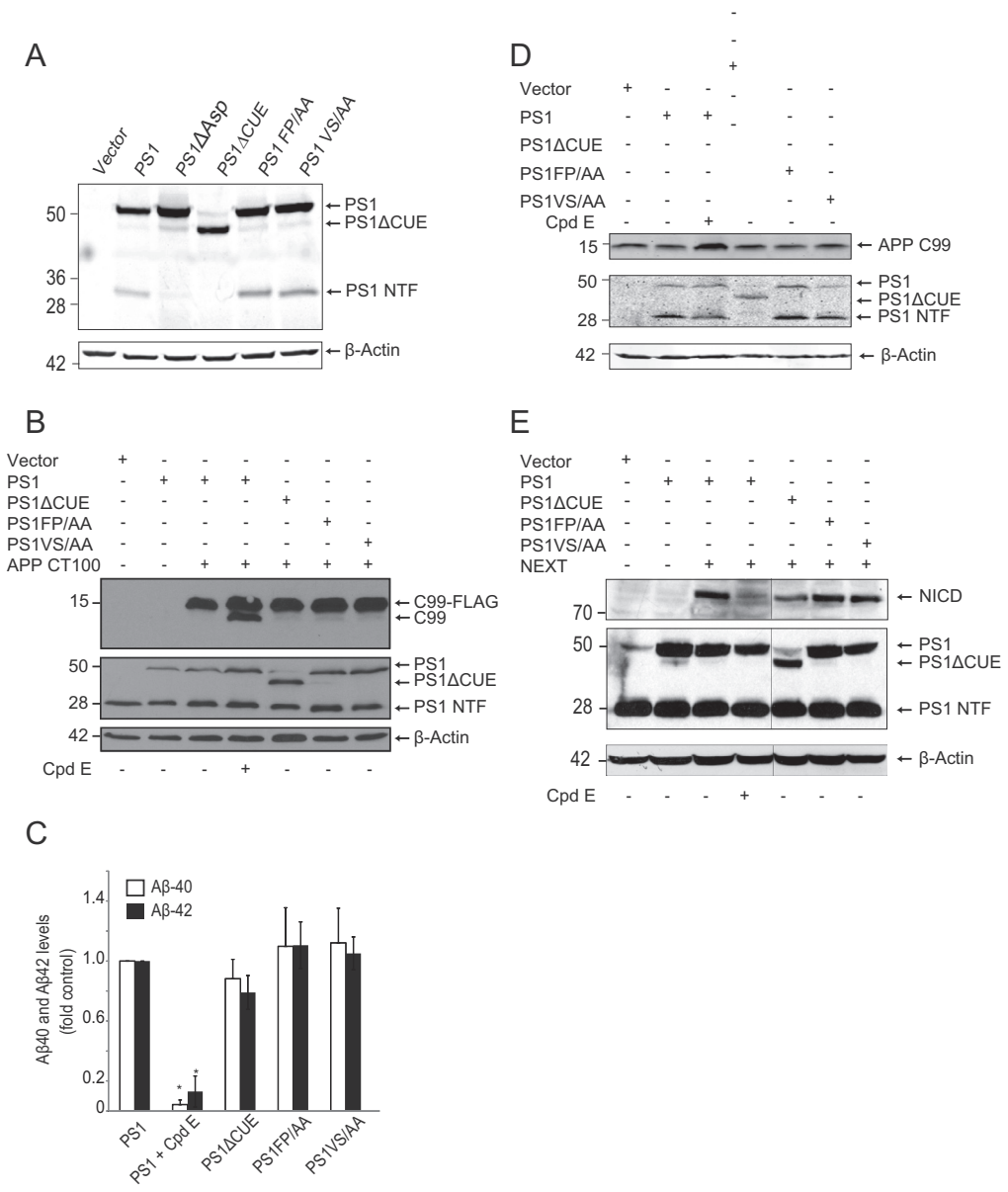

Fig. 4. Presenilins CUE domain is dispensable for γ-secretase activity. (A) Western blot analysis of total cell lysates from presenilin-deficient MEFs transiently expressing pcDNA3.1 (Vector) wild type PS1, catalytically inactive PS1D257A/D385A (PS1ΔAsp) mutant, PS1ΔCUE, PS1F283A/P284A or PS1S310A/V309A. (B) HEK293T cells were transfected with APP CT100-FLAG and co-transfected with pcDNA3.1 (Vector) wild type PS1, PS1ΔCUE, PS1F283A/P284A or PS1V309A/S310A. Twelve hours after transfection, selected cell cultures were treated with Compound E (50 nM) as indicated. Cells were harvested 24 h after transfection and lysates were subjected to western blot analysis with anti-APP, anti-PS1 and anti-β-actin antibodies. (C) Culture medium was collected from HEK293T cells transfected with APP CT100-FLAG and co-transfected with pcDNA3.1 (Vector) wild type PS1, PS1ΔCUE, PS1F283A/P284A or PS1V309A/S310A, and subjected to ELISA for Aβ40 and Aβ42. Data is presented by means ± S.E.M. of three independent experiments. $p < 0.05$ was considered statistically significant. (D) Western blot analysis of cell lysates from presenilin-deficient MEFs transiently expressing pcDNA3.1 (Vector) wild type PS1, PS1D257A/D385A, PS1ΔCUE, PS1F283A/P284A or PS1S310A/V309A. (E) HEK293T cells were transfected with NEXT expression plasmid and co-transfected with pcDNA3.1 (Vector) wild type PS1, PS1D257A/D385A, PS1ΔCUE, PS1F283A/P284A or PS1V309A/S310A. Twelve hours after transfection selected cell cultures were treated with Compound E (50 nM) as indicated. Cells were harvested 24 h after transfection. Lysates were subjected to SDS–PAGE and immunoblotted with anti-cleaved Notch, anti-PS1 and anti-β-actin antibodies.

NICD formation is still observed. This data shows that deletion of the CUE domain of PS1 or mutagenesis of key motifs within the PS1 CUE domain has no effect on the γ-secretase mediated cleavage of Notch.

4. Discussion

Here we describe the presence of a CUE ubiquitin-binding domain in the hydrophilic loop domain of presenilins, which facilitates an interaction between PS1 and K63-linked polyubiquitin chains. Despite the significant importance of presenilins as the catalytic component of γ-secretase protease complexes, its structure and posttranslational regulation are poorly understood. In addition to endoproteolysis, presenilins are also regulated by diverse posttranslational modifications including phosphorylation [39,47,48] and ubiquitination [31,34], which alter the functions of presenilins and their interaction with other proteins [29]. These modifications are not only essential for the stability and activation of presenilins, but are also important for the protease assembly and activity of γ-secretase complexes. For instance, SEL-10, a member of the SCF (Skp1-Cdc53/CUL1-F-box protein) E2–E3 ubiquitin ligase family was shown to interact with and

enhance PS1 ubiquitination, and alter the cellular levels of PS1 holoprotein and its NTF/CTF heterodimers [34]. In contrast, we have also reported the ubiquitination of PS1 and PS2 by another E3 ligase, tumor necrosis factor receptor-associated factor 6 (TRAF6) [35], which alters the stability of PS1 holoprotein and its function in the regulation of calcium homeostasis, independent of γ-secretase protease activity.

Thus, the identification of a CUE domain represents an important advancement in the understanding of the posttranslational activity in these biologically and therapeutically important proteins. The CUE domain is structurally related to the ubiquitin-binding UBA domain and is strictly comprised of a three-helix bundle structure (Fig. 1). However, the amino acid sequence of CUE domains does differ from the consensus amino acid sequence, suggesting that the structural integrity of the CUE domain rather that amino acids sequence is the primary determinant for interaction with different ubiquitin modifications. Within the CUE domain, a conserved MFP motif in α-helix1 and a less conserved LL motif in α-helix 3 have been shown to enable interaction with the conserved hydrophobic patch of ubiquitin. While deletion of the entire PS1 CUE domain abolished binding to ubiquitin, mutation of the MFP motif in PS1 did not significantly disrupt interaction with ubiquitin. However, mutagenesis of the PS1 VS sequences that corresponds to the LL motif in α-helix 3 did disrupt binding to ubiquitin. It was surprising that mutation of the MFP motif in PS1 did not disrupt interaction with ubiquitin, as is the case for many published CUE domain-containing proteins. These discrepancies suggest that the PS1 CUE domain may bind to ubiquitin in a different manner. Site-directed mutagenesis and deletion of residues predicted to have an important role in non-covalent ubiquitin binding [43] revealed and highlight the biological importance of this domain in presenilins. First, using the CUE domain deletion and missense CUE mutants demonstrated loss in ability of PS1 to selectively bind to K63-linked polyubiquitin chains. Second, missense CUE mutants that prevented binding to ubiquitin, did not affect endoproteolysis of PS1, suggesting that formation of PS1NTF/CTF heterodimers does not require an intact CUE domain, but suggest that the presence of the CUE domain may be important for a PS1 holoprotein function. Third, mutation of the CUE domain did not alter PS1-dependent γ-secretase cleave of APP or Notch, nor generation of Aβ40/42 peptides, suggesting that the PS1 CUE domain is not necessary for γ-secretase activity.

Recognition of ubiquitin and polyubiquitin chains by ubiquitin-binding domains (UBDs) is vital for ubiquitin-mediated signaling pathways [9]. The presenilins regulate critical proteins and biological functions via γ-secretase dependent and independent mechanisms [49]. Based on other studies that have elucidated the function of CUE domains on other proteins, the presence of the CUE domain in presenilins leads to at least three possible functions of this CUE domain; ubiquitination of presenilin enables the CUE domain to recognize and non-covalently interact with presenilin itself (intraprotein interaction); ubiquitination of presenilin leads to the formation of a presenilin homodimer enabled by interaction between the CUE domain and ubiquitinated presenilin on an adjacent holoprotein, or the CUE domain of presenilins facilitates its interaction with other ubiquitin-modified proteins (interprotein interaction).

Taken together our results highlight a function for non-covalent ubiquitin signaling in the regulation of the presenilins, add new insight into the structure and function of the presenilins and have important implications for cell signaling and Alzheimer's disease.

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